CONTROL OF CHOLESTEROL CATABOLISM IN RAT LIVER

Maureen J. G. Brown

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This thesis was composed by myself and the results described therein are the product of my own work.

Maureen J. G. Brown

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SUMMARY

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This study is concerned with the mechanisms which control the activity of rat liver microsomal cholesterol 7α-hydroxylase. This enzyme is thought to catalyse the rate limiting step in the catabolism of cholesterol to bile acids. Previous assays used to measure the enzyme activity have involved the addition of a tracer amount of radioactive cholesterol to rat liver microsomal suspensions and measurement of the rate of formation of radioactive product. This procedure assumes that the added tracer equilibrates rapidly with all the endogenous cholesterol and that the total substrate pool size is the same in different microsomal preparations. An assay has been developed which measures the total mass of cholest-5-ene-3β, 7α-diol (7α-hydroxycholesterol) formed during incubations. This method does not involve any of the assumptions inherent in the radioactive tracer assay. The procedure involves purification of the 7α-hydroxycholesterol by thin layer chromatography, followed by acetylation and finally computation by gas liquid chromatography. Using this method it was shown that radioactive tracer cholesterol added in acetone solution equilibrates rapidly with virtually all the endogenous microsomal cholesterol. This gas liquid chromatographic assay was used to investigate the activity of rat liver microsomal cholesterol 7α-hydroxylase under various physiological conditions. The activity of the enzyme in control rats was found to depend on the diet on which the animals were maintained. The activity of the

enzyme was significantly greater when animals were fed the commercial pellet diet than when they were fed a semi-synthetic soft diet. Cholesterol 7α -hydroxylase activity was compared with the rate of hepatic cholesterol biosynthesis measured using (1-^{14}C) acetate. Cholesterol feeding, which inhibits cholesterol synthesis from acetate in rat liver, was shown to stimulate cholesterol 7α -hydroxylase to a small but significant extent. Starvation, which inhibits hepatic cholesterol synthesis, also inhibited cholesterol 7α -hydroxylase. However, biliary drainage was found to stimulate cholesterol 7α -hydroxylase activity in the starved rat. Cholestyramine feeding, which inhibits the absorption of both bile acids and cholesterol from the gut, stimulated both hepatic cholesterologenesis from acetate and cholesterol 7α -hydroxylase, in agreement with previous reports. Tomatine feeding, which inhibits the intestinal absorption of cholesterol but not bile acids, stimulated hepatic cholesterologenesis but not cholesterol 7α -hydroxylase. These results suggest that hepatic cholesterol biosynthesis and degradation are controlled by independent mechanisms.

Rat liver microsomal cholesterol 7α -hydroxylase requires NADPH and molecular oxygen and involves an electron transport system having cytochrome P450 as the terminal oxidase. A large variety of drugs and foreign compounds are oxidised in liver microsomes by a similar mechanism. Experiments have been performed which show that the 7α -hydroxylation of cholesterol and the oxidation of drugs are controlled by different mechanisms <u>in vivo</u>.

Evidence has been presented which suggests that this may be due to the presence of different rate limiting steps in the enzyme systems. It is suggested that the rate limiting step in the 7α -hydroxylation of cholesterol might be the formation of an enzyme-cholesterol complex.

The specificity of the cholesterol 7α -hydroxylase enzyme has been investigated by comparing the liver microsomal oxidation of cholesterol with that of several sterols having a similar structure to cholesterol. The substrates used were 3β -hydroxyandrost-5-ene-17-one, 3β -hydroxypregn-5-ene-20-one, androst-5-ene-3β -ol and pregn-5-ene-3β -ol. All of these sterols were metabolised to one or more products when incubated with rat liver microsomes, NADPH and oxygen. The former three sterols gave a Type I difference spectrum with liver microsomes suggesting that they might be oxidised by a mechanism involving cytochrome P450. Pregn-5-ene-3β -ol, however, gave no difference spectrum and its oxidation was inhibited by β -mercaptoethylamine suggesting that a peroxidative mechanism might be involved. Treatment of rats with cholestyramine, which increased the activity of liver microsomal cholesterol 7α -hydroxylase, had no effect on the rate of oxidation of any of the four sterols studied. This suggests that either these sterols are oxidised by different enzyme systems from that involved in cholesterol 7α -hydroxylation, or that different rate limiting steps are involved in the overall mixed function oxidase enzyme complex.

CONTENTS

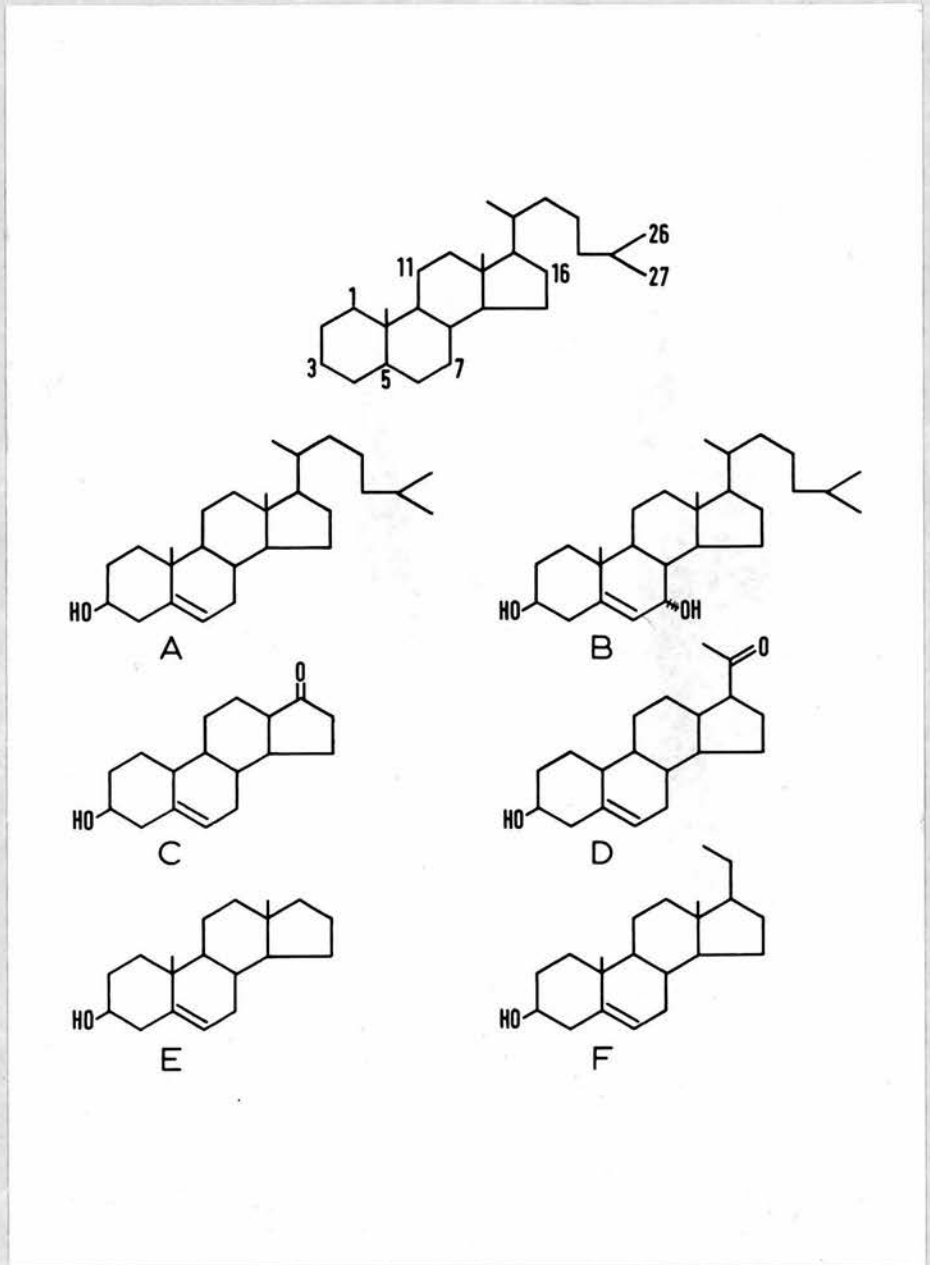


Figure 1 The trivial and systematic names of some steroids mentioned in this text; (A) cholest-5-ene-3β-ol, cholesterol; (B) cholest-5-ene-3β,7α-diol, 7α-hydroxycholesterol; (C) 3β-hydroxyandrost-5-ene-17-one, dehydroepiandrosterone; (D) 3β-hydroxypregn-5-ene-20-one, pregnenolone; (E) androst-5-ene-3β-ol; (F) pregn-5-ene-3β-ol.

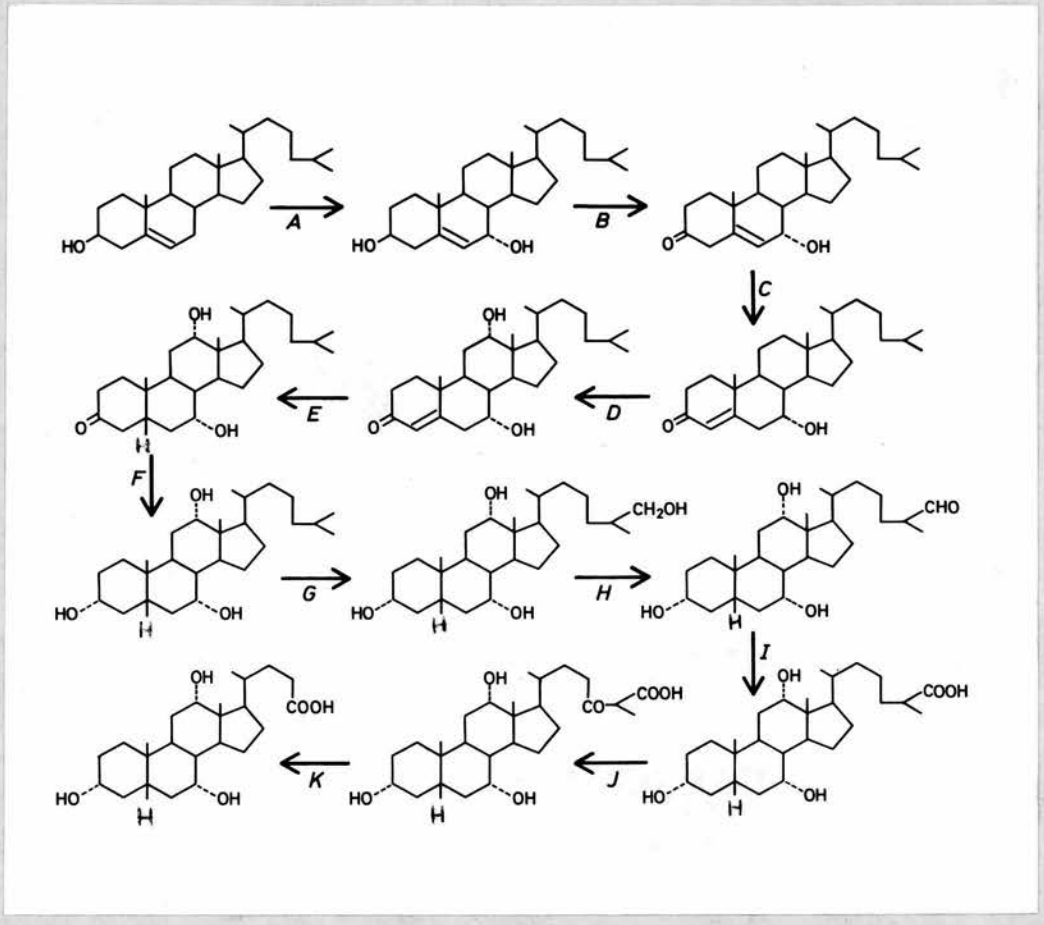


Figure 2 The suggested pathway for cholesterol conversion to cholic acid in rat liver.

SECTION 1

INTRODUCTION

The major quantitative pathways for cholesterol catabolism in mammals are bile acid synthesis and the excretion of faecal neutral sterols (Siperstein et al 1952). Elimination of sterols via skin and urine and the synthesis of steroid hormones are quantitatively of minor importance. The liver is the only organ known to convert cholesterol into bile acids, the end products being the C_{24} compounds cholic and chenodeoxycholic acids (review by Elliot and Hyde 1971). The major enzymic pathway for cholic acid formation in rat liver is thought to be that shown in Fig.2, while chenodeoxycholic acid is formed by the same route, but omitting the 12α-hydroxylation (step D).

These primary bile acids are conjugated with either taurine or glycine and in the form of the sodium salts they are secreted via the bile duct into the duodenum. In the gut, bile salts are functional in the emulsification of dietary fat prior to its digestion by pancreatic lipase, in the activation of this lipase and in the efficient absorption of dietary lipids by the cells of the intestinal mucosa.

Bile salts are further metabolised by micro-organisms present in the caecum and terminal ileum. Bile acids extracted from faeces are entirely unconjugated and several species of Clostridium and Enterococcus have been shown to effect the hydrolysis of the peptide bond of the taurine and glycine conjugates. Cholic and chenodeoxycholic acids can be 7-dehydroxylated to deoxycholic and lithocholic acids respectively by certain organisms, for example an

anaerobic Lactobacillus. Other transformations which occur are the oxidation of hydroxyl groups to the ketones and reduction of the ketones to give both α and β-hydroxylated epimers (review by Danielsson and Tchen 1968). The caecum and the lower part of the ileum adsorb certain of these secondary bile acids along with unchanged primary bile acids. These return via the portal blood to the liver where they are further metabolised and resecreted, constituting an enterohepatic circulation of bile salts. In the rat, approximately 90% of bile salts are reabsorbed from the intestine, but faecal losses are compensated by continual synthesis from cholesterol in the liver, thus keeping the total circulating pool relatively constant. It has been calculated that in the rat this pool is circulated ten to thirteen times per day (Shefer et al 1969). The role of intestinal micro-organisms in regulating bile acid metabolism has been investigated usingrats raised under germ-free conditions (Gustaffson et al 1957). It was found that the half-life of cholic acid was three to five-fold greater in germ-free rats than in conventional animals. Alterations in the rate of bile acid metabolism produced by administering different dietary regimens might be secondary to changes in the composition of the intestinal flora. A dietary effect was reported by Portman and Murphy (1958) who found that the bile acid excretion rate in rats with a biliary fistula was considerably lower when the animals were fed a purely synthetic diet than when they were fed a commercial diet (Purina rat chow).

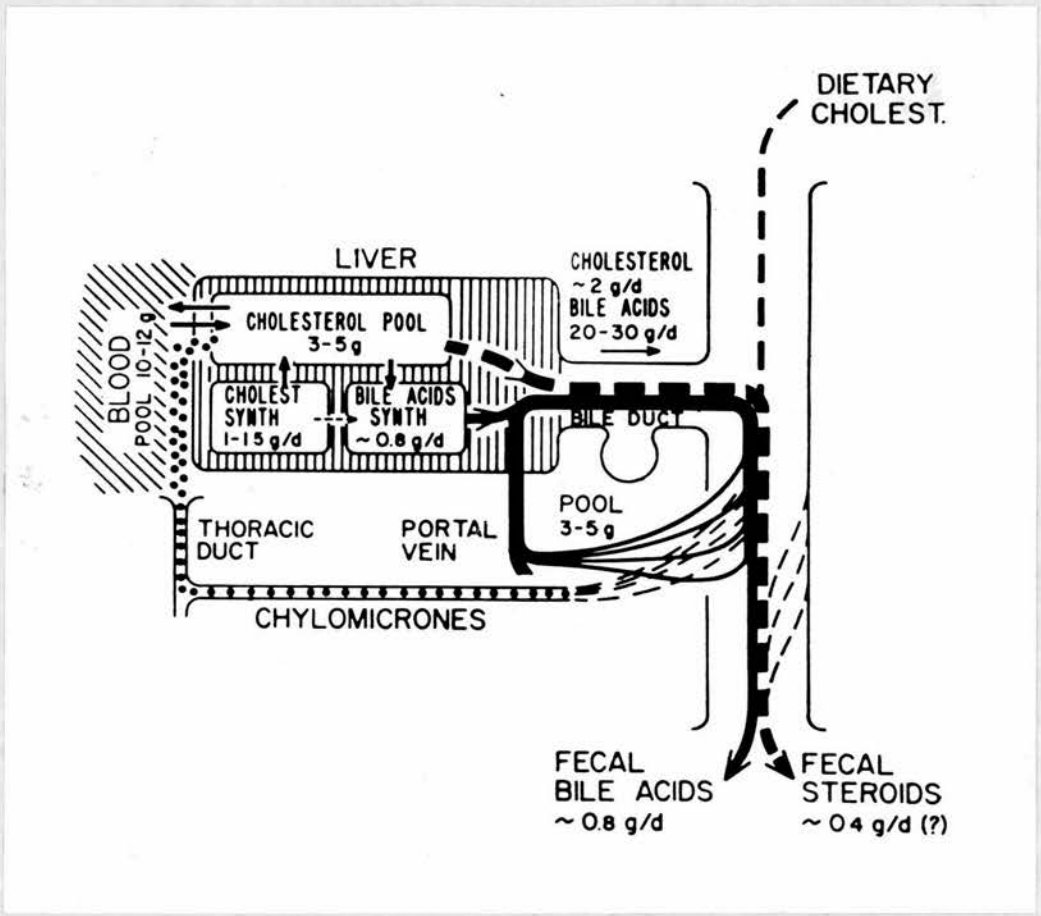


Figure 3 The enterohepatic circulation of cholesterol and bile acids in man (from Bergström, 1959).

Other compounds which undergo enterohepatic circulation are the bile pigments, steroid metabolites (review by Taylor 1971), and to a lesser extent, cholesterol. Cholesterol is secreted from the liver via the bile duct to the small intestine where it mixes with dietary cholesterol. A portion of this is reabsorbed via the lymphatic system and returns to the liver via the systemic circulation incorporated into chylomicrons. The neutral sterol fraction of faeces is composed of cholesterol, other steroids of endogenous origin such as 5α-cholestan-3β-ol (cholestanol) and microbial degradation products of cholesterol such as 5β-cholestan-3β-ol (coprostanol). The enterohepatic circulation of cholesterol and bile salts is shown diagrammatically in Fig. 3.

If the circulation of bile salts is prevented, either by inserting a cannula into the common bile duct and draining the bile externally, or by feeding the anion exchange resin cholestyramine which binds bile salts at the pH of the small intestine, and prevents their reabsorption, the liver responds by increasing the synthesis of bile acids by as much as ten-fold (Eriksson 1957; Bergström and Danielsson 1958). This suggests that there is a feedback control by bile acids on their own synthesis. This hypothesis is supported by the results of Grundy et al (1966) who reported that the administration of bile acids to man suppressed endogenous bile acid production, and by the results of Shefer et al (1969) who showed that the infusion of sodium taurocholate into rats with a biliary fistula prevented the increase in bile acid biosynthesis. Also

ileal resection, which prevents the complete reabsorption of bile acids, results in an increase in bile acid biosynthesis (Playoust *et al* 1965).

Linstedt (1957) first suggested that the initial step in the transformation of cholesterol to bile acids was the 7α-hydroxylation reaction and circumstantial evidence indicates that this enzyme may be rate-limiting in bile acid formation. Biliary diversion in the rat results in an increase in the activity of the cholesterol 7α-hydroxylase enzyme (Danielsson *et al* 1967; Shefer *et al* 1968; Boyd *et al* 1969). Thus it seems likely that the feedback control of bile acid biosynthesis takes place at the level of this enzyme. After complete drainage of bile salts from the liver of a bile fistula rat, there is a lag period of several hours before the increase in bile acid biosynthesis occurs. This suggests that the feedback control is not exerted by a direct effect of bile acids on the enzyme, but rather that the induction of enzyme activity is due to the synthesis of new enzyme protein. This is supported by the results of Einarsson and Johansson (1968a) who demonstrated that administration of inhibitors of protein biosynthesis prevented the induction of cholesterol 7α-hydroxylase activity in the bile fistula rat.

These studies provide good evidence that prevention of the resorption of bile acids from the gut accelerates bile acid production, probably by increasing the activity of the enzyme cholesterol 7α-hydroxylase. However, the mechanism whereby this feedback is exerted is obscure. It was shown by Myant and Eder in 1961 that the increase in bile acid

production following the insertion of a biliary fistula into rats was preceded by an increase in hepatic cholesterol biosynthesis. Danielsson et al (1967) and Weis and Dietschy (1969) have since demonstrated that the enzyme most affected by this procedure is probably β-hydroxy β-methyl glutaryl coenzyme A reductase (HMG CoA reductase) which is the major rate-limiting enzyme in cholesterol biosynthesis in the liver. Thus there appears to be a dual feedback mechanism in the regulation of bile acid production. Biliary diversion is accompanied by an increase in the activity of both hepatic HMG CoA reductase and cholesterol 7α-hydroxylase. This has led to a great deal of discussion as to the regulatory factors involved in hepatic cholesterol biosynthesis and bile acid formation (Dietschy and Wilson 1970; Hamprecht et al 1971). The situation is complicated by the fact that bile salts are required for the efficient intestinal absorption of cholesterol, hence diversion of bile salts from the small intestine prevents the feedback of cholesterol to the liver. It is not yet clear as to whether hepatic cholesterol biosynthesis and degradation are both regulated by absorbed bile acids or absorbed cholesterol or whether each system is regulated by an independent mechanism. The interrelationships between hepatic HMG CoA reductase and cholesterol 7α-hydroxylase are discussed in Section 4. Some experiments are described which investigate the activity of rat liver cholesterol 7α-hydroxylase in relation to the rate of hepatic cholesterologenesis.

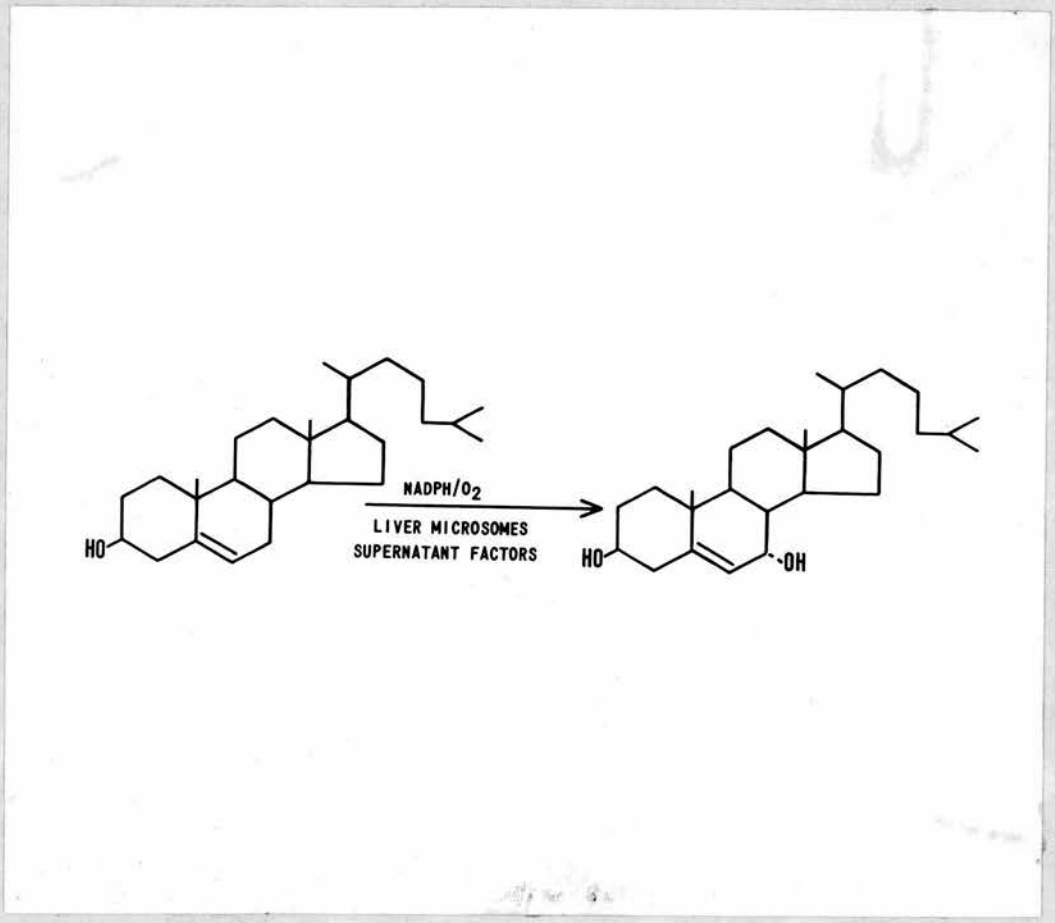


Figure 4 The 7α -hydroxylation of cholesterol.

The properties of the rat liver cholesterol 7α-hydroxylase enzyme have been extensively investigated in this and in other laboratories (Mitton et al 1971; Shefer et al 1968). It is located in the microsomal fraction of rat liver and has an absolute requirement for NADPH, oxygen and certain supernatant factors (Fig. 4). The enzyme has been assayed by incubating a tracer amount of radioactive cholesterol with liver microsomes and suitable cofactors, followed by extraction, separation and quantitation of the radioactive sterols. It has been shown that without the presence of 105,000 g liver supernatant, several products are formed, namely 3β-hydroxycholest-5-ene-7-one (7-ketocholesterol), cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol), cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol) and cholestan-3β,5α,6β-triol. However in the presence of cell supernatant and excess NADPH there is selective formation of 7α-hydroxycholesterol (Mitton et al 1971). The supernatant can be replaced by β-mercaptoethylamine (Scholan and Boyd 1968) or by glutathione and manganous ions (Grimwade 1971). The formation of these other products, 'autoxidation products' as they have been termed, occurs under the same conditions as liver microsomal lipid peroxidation and it has been suggested that they are formed by a similar mechanism (Grimwade et al 1971). The possible mode of formation of these products is shown in Fig. 5.

The use of the radioactive tracer assay in investigating the metabolism of cholesterol poses certain problems due to the presence of appreciable amounts of endogenous

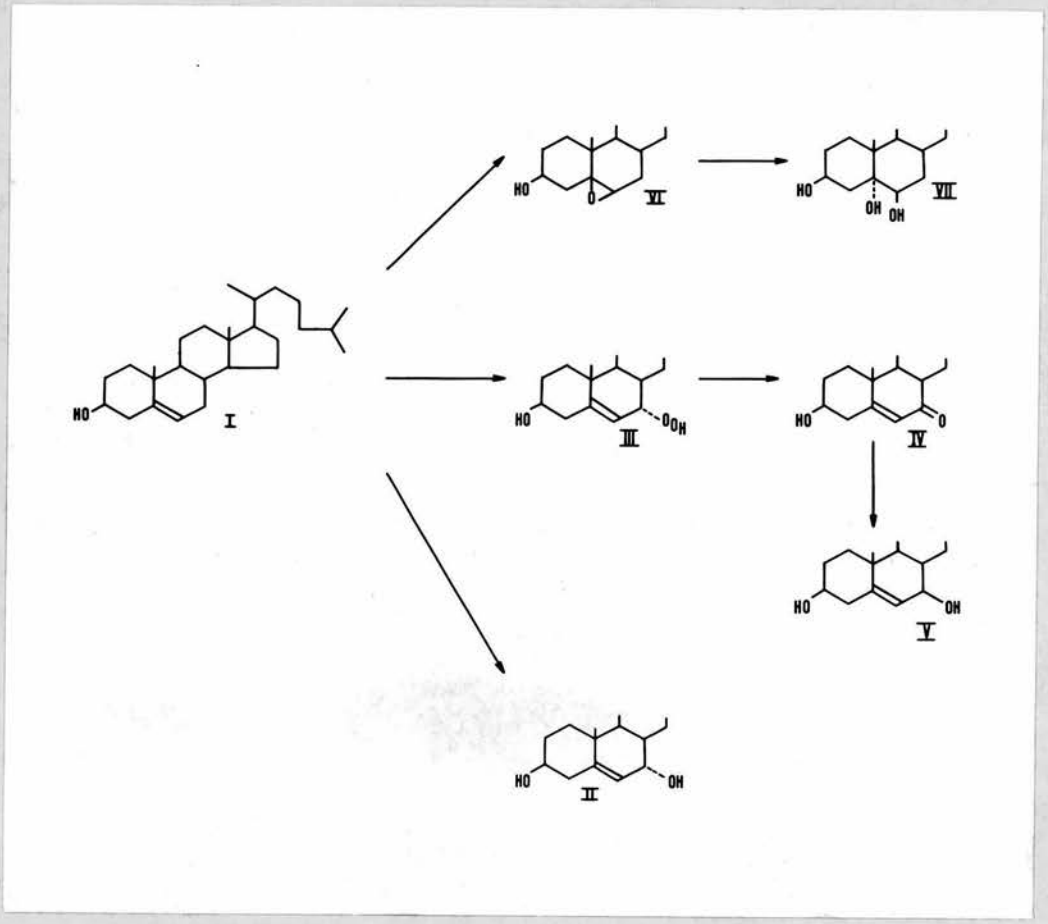


Figure 5 Suggested mechanism for the formation of cholesterol 'autoxidation products' by rat liver microsomes (from Mitton et al 1971).
(I) cholesterol, (II) 7α-hydroxycholesterol, (III) cholesterol 7α-hydroperoxide, (IV) 7-ketocholesterol, (V) 7β-hydroxycholesterol, (VI) cholesterol-5,6-oxide, (VII) cholestan-3β, 5α,6β-triol.

cholesterol in liver sub-cellular fractions. It is possible that liver microsomal cholesterol is present in discrete heterogeneous pools and that not all of this cholesterol acts as substrate for the cholesterol 7α-hydroxylase enzyme. If the added tracer does not equilibrate immediately with all the endogenous cholesterol, then the use of the radioactive tracer assay could lead to erroneous results, in particular when it is used to measure the activity of the enzyme under different *in vivo* treatments and dietary regimens. It is therefore desirable to establish a means of measuring directly the amount of 7α-hydroxycholesterol produced, thus obviating the need for assumptions regarding substrate pool sizes or equilibration of tracer. One of the aims of this work was to develop such a method, hence providing a more reliable means of investigating the physiological control of the enzyme.

Rat liver microsomal cholesterol 7α-hydroxylase requires NADPH and oxygen and thus falls into the class of enzymes known as 'mixed function oxidases' (Mason 1957). These reactions require a source of reducing equivalents and molecular oxygen. One of the atoms in the oxygen molecule is incorporated into the substrate and the other is reduced to water. These enzymes involve an oxygen activating component, and in a number of cases this component has been shown to be cytochrome P450, so termed because of the spectrum of the reduced carbon monoxide complex which has a maximum absorption at 450 nm. The presence of this cytochrome was first demonstrated in liver microsomes by Klingenberg and Garfinkel independently

in 1958, but its presence has since been shown in a number of other tissues, notably adrenal cortex. Enzymes which involve this cytochrome as the oxygen activating species are characterised by their inhibition by carbon monoxide, the inhibition being maximally reversible by monochromatic light at 450 nm. The first enzyme demonstrated to possess this property was the steroid 21-hydroxylase from bovine adrenal cortical microsomes (Estabrook et al 1963), but since then many other enzymes have been implicated. These include the 11β-hydroxylation of deoxycorticosterone (Omura et al 1965) and the side-chain cleavage of cholesterol to pregnenolone (Simpson and Boyd 1967) in bovine adrenal cortex mitochondria.

The liver microsomal mixed function oxidase system involving cytochrome P450 appears to catalyse the oxidation of a large number of substrates of widely differing chemical structures (review by Conney 1967). These include barbiturates such as phenobarbitone, carcinogens such as benzpyrene and endogenous steroids such as testosterone. Liver microsomal cytochrome P450 is thought to be reduced by an electron transport system involving NADPH and a flavoprotein (Orrenius and Ernster 1964). Cytochrome b_5, another cytochrome present in liver microsomes, has been suggested to play a role in the reduction of cytochrome P450 (Hildebrandt and Estabrook 1971).

The liver microsomal cytochrome P450-dependent enzymes are induced following the administration to animals of certain oxidisable substrates. The induction mechanism appears to be non-specific in that the administration of

one compound results in an enhancement of the ability to oxidise in addition a large number of other substrates (Conney 1967). The induction of enzyme activity is paralleled by an increase in the content of cytochrome P450 in the liver, suggesting that this component might be rate-limiting in the overall hydroxylation mechanism.

There is evidence to suggest that there are two forms of cytochrome P450, one of which is induced on administration of polycyclic hydrocarbons such as 3-methylcholanthrene, and another form which is inducible by barbiturates, for example phenobarbitone (Sladek and Mannering 1966). These forms are thought to differ in the spin state of the haem moiety. In the high spin state the five electrons in the 5d orbitals of the haem iron atom are unpaired, whereas in the low spin state there is only one unpaired electron. The two forms can be distinguished by visible spectroscopy and by electron paramagnetic resonance (Hildebrandt et al 1968; Jefcoate and Gaylor 1969). 3-Methylcholanthrene induces a form of the cytochrome having the properties of a high spin haemoprotein, whereas phenobarbitone induces both the low spin and the high spin forms. It is not known whether these are two distinct haemoproteins or interconvertible forms of the one haemoprotein. Cytochrome P450 is thought to be the substrate binding site of the mixed function oxidase system. When oxidisable substrates are added to oxidised suspensions of liver microsomes under aerobic conditions, a characteristic difference spectrum is observed (Remmer et al 1966) which is thought to be due to spin-state transitions of the haem moiety of cytochrome P450

(Hildebrandt *et al* 1968). Compounds producing this effect have been classified as Type I or Type II according to the characteristics of the difference spectrum. Type I compounds produce a difference spectrum having a maximum absorption at approximately 392 nm and a minimum at approximately 410 nm whereas the difference spectrum produced by Type II compounds has a minimum at 390 nm and a maximum at approximately 420 nm.

It has been shown in this laboratory that cytochrome P450 participates in the 7α-hydroxylation of cholesterol by rat liver microsomes (Boyd *et al* 1971). In this respect cholesterol 7α-hydroxylation is similar to the oxidation of drugs and steroids by liver microsomes. However cholestyramine feeding, which enhances cholesterol 7α-hydroxylase activity, had no effect on liver microsomal cytochrome P450 levels (Boyd *et al* 1969) suggesting that this component is not rate-limiting in the hydroxylation of cholesterol by liver microsomes. In Section 5 some experiments are described which investigate the relationship of the cholesterol 7α-hydroxylase enzyme system to the other mixed function oxidases of rat liver microsomes, with particular reference to the nature of the rate-limiting step.

A number of endogenous steroid substrates are hydroxylated by liver microsomes and it is of interest to establish whether these are metabolised by the same enzyme system as cholesterol. The specificity of liver microsomal cholesterol 7α-hydroxylase has been studied by investigating the metabolism of a variety of steroid substrates and comparing their oxidation with that of cholesterol.

In summary, the aims of this research have been to investigate the mechanisms whereby the rat liver microsomal cholesterol 7α-hydroxylase is controlled *in vivo*. The regulation of this enzyme has been compared with that of hepatic cholesterol biosynthesis and with the cytochrome P450-dependent mixed function oxidase system of rat liver microsomes.

SECTION 2

MATERIALS AND METHODS

A. Animals and Their Diets

The animals used in these experiments were rats of the Wistar strain weighing 150-250 g and were bred in the animal unit of this department. Unless otherwise stated, male rats were used.

Control diets used were a pellet diet, 'Oxoid' no.86 rat cake (75% whole meal flour, 20% fish and bone meal and 5% dried yeast) and a soft diet (70% whole meal flour, 25% skimmed milk powder and 5% dried yeast).

The various dietary regimens used in some experiments involved the incorporation of the additive into the soft diet. Cholestyramine ('Cuemid', Merck, Sharp and Dohme) was administered at a dose of 4% by weight in the diet for at least five days. Tomatine (Koch-Light) was sieved before mixing with the diet (1% by weight) and was administered for six to eight days. In cholesterol feeding experiments cholesterol (1% by weight) was dissolved in olive oil (10% by weight) and mixed with the diet. Controls received 10% olive oil in soft diet. Starved animals were kept without food for 48 hours prior to death but were given water to drink ad libitum.

B. Bile Duct Cannulations

A polyethylene cannula was inserted andtied into the common bile duct and held in place outside the animal by surgical tape. The animals were held under ether anaesthesia throughout this procedure. They were kept in special restriction cages and given an electrolyte solution of NaCl

(0'7%), $NaHCO_3$ (0'25%), KCl (0'037%) to drink. Controls were subjected to the same surgical stress but no cannula was placed in the bile duct and they were given water to drink.

C. Phenobarbitone Treatment

Sodium phenobarbitone was administered to rats in their drinking water at a concentration of 1 mg/ml. This treatment was continued for six to eight days.

D. 3-Methylcholanthrene Treatment

3-Methylcholanthrene was suspended in corn oil (Mazola) and injected intraperitoneally at a dose of 20 mg/kg body weight. Control animals received the same volume of corn oil (0'5 ml). The animals were injected in this manner once per day for three days and were killed on the fourth day.

E. Cycloheximide Treatment

Cycloheximide was dissolved in 0'9% saline and the animals received a single intraperitoneal injection at a dose of 5 mg/kg body weight. Controls received the same volume of saline (0'2 ml).

F. Preparation of Liver Microsomes

The animals were anaesthetised with ether and the livers were immediately excised and weighed. In certain experiments in which it was necessary to remove as much haemoglobin as possible the liver was first perfused *in situ* with 0'154 M KCl through the portal vein. All subsequent procedures were performed at 0-4°C. The liver was minced with scissors, then homogenised in 0'154 M KCl (20%

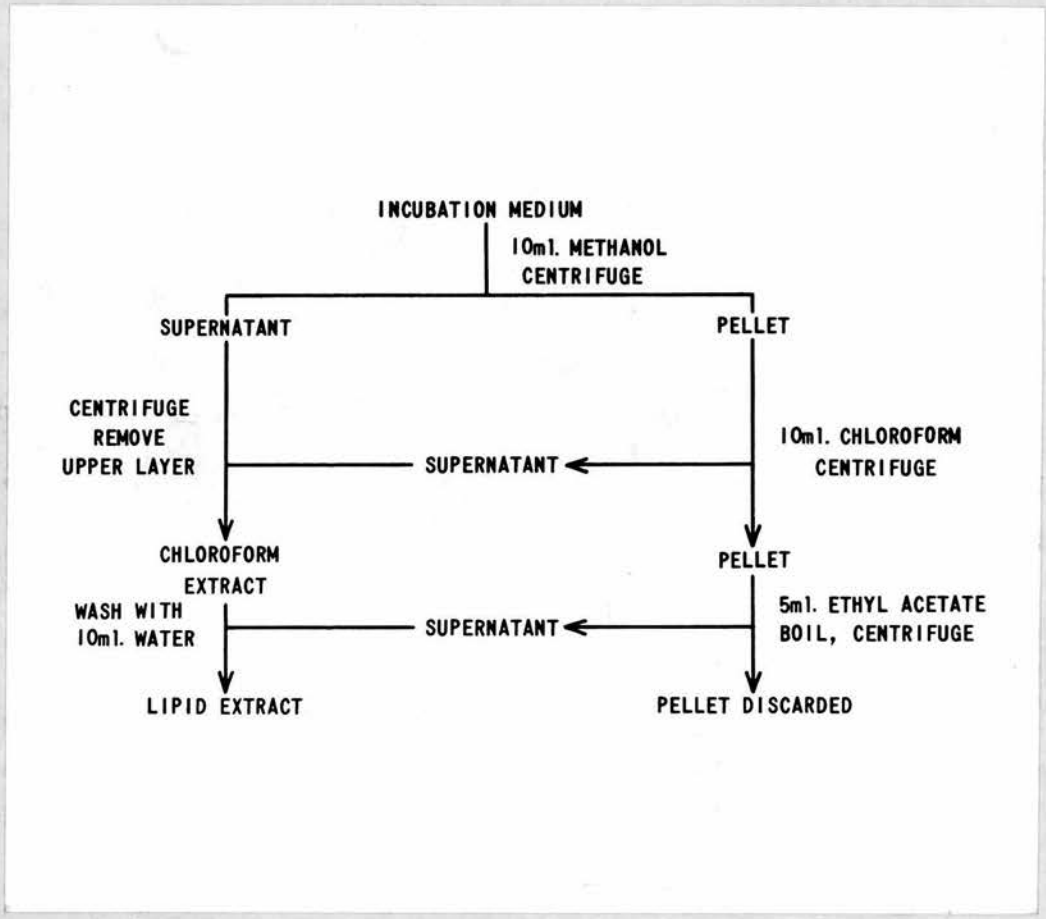


Figure 6 Procedure for the extraction of steroids from rat liver microsomal incubations.

w/v) by three passes of a loosely-fitting Teflon/glass homogeniser. The crude homogenate was centrifuged at 18,000 g for 15 minutes to remove nuclei, unbroken cells, cell debris and the mitochondrial fraction. The 18,000 g supernatant was then centrifuged at 100,000 g for 1 hour to sediment the endoplasmic reticulum as the microsomal fraction. The microsomal pellet was suspended in 0'1 M sodium phosphate buffer pH 7'4, so that 1 ml of the final suspension was equivalent to 1 g wet weight of liver.

G. Radioactive Tracer Assay for Cholesterol 7α-Hydroxylase

Each incubation contained a microsomal suspension derived from 1 g of liver buffered with a total of 2 ml of 0'1 M phosphate buffer pH 7'4. NADPH was added in the form of a generator consisting of NADP (5 µmoles), glucose 6-phosphate (50 µmoles) and glucose 6-phosphate dehydrogenase (1 unit) in 1 ml of water. (4-^{14}C) cholesterol, purified by thin layer chromatography prior to use, was added to the incubation medium in 0'05 ml of acetone (0'1 µC, specific activity 60 mC/mM). The incubation medium also contained 10 mM β-mercaptoethylamine. Water was added to give a total volume of 7'0 ml.

Incubations were conducted in air for 1 hour in a shaking water bath (100 oscillations/minute) at 37^{o}C. The optimal conditions for enzyme activity have previously been established in this laboratory (Mitton et al 1971). The reaction was stopped by the addition of 10 ml of methanol. Lipid was extracted from the tissue according to the procedure shown in Fig. 6. The extract was concentrated and applied to a thin layer chromatography (TLC) plate of

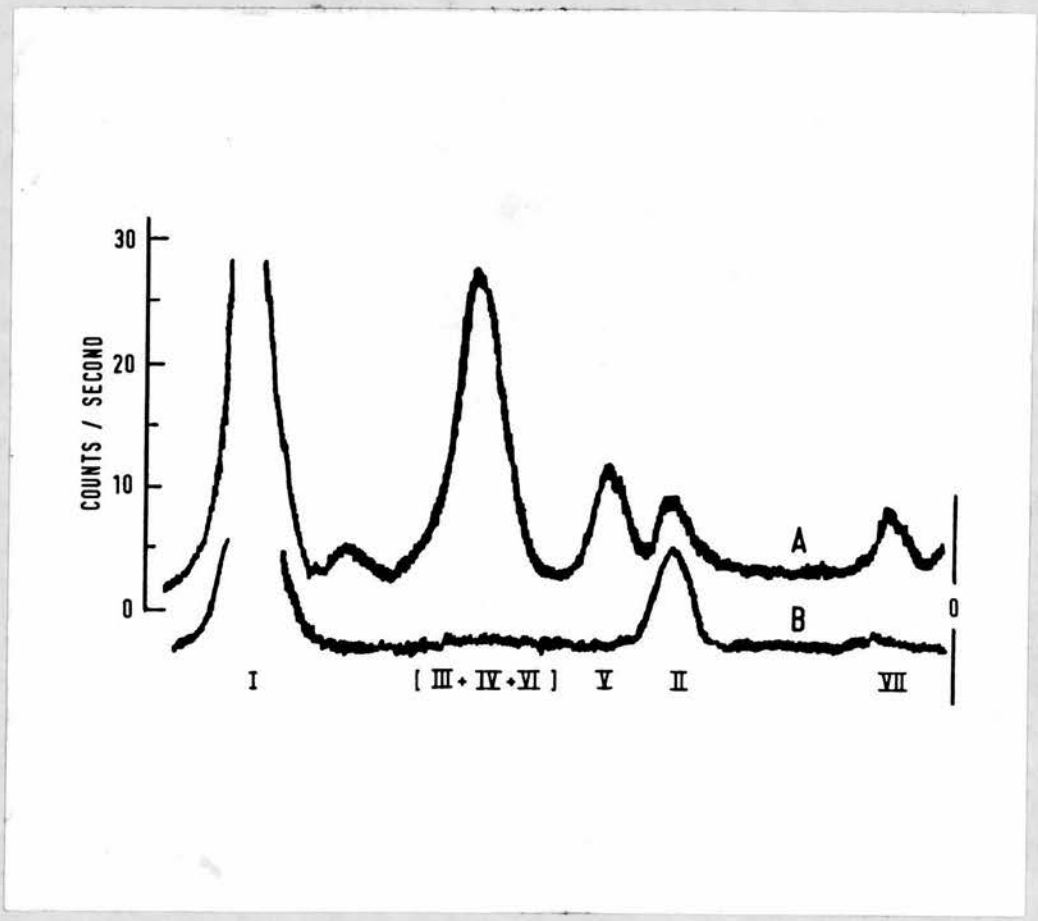


Figure 7 Radioactive scans of thin layer plates of the lipid extracts obtained after incubation of (4-^{14}C) cholesterol with rat liver microsomes in the absence (A) and the presence (B) of 10 mM β-mercaptoethylamine. The products are identified in Fig. 5.

silica gel H (Merck) which was developed in a solvent system of benzene:ethyl acetate: :7:13. The radioactive compounds were located using a Panax thin layer scanner and identified by their R_F values. The products obtained from cholesterol have been characterised in this laboratory (Mitton et al, 1971). The products were quantified by removing the silica gel into liquid scintillation vials and measuring the radioactivity in a Packard Tri-Carb liquid scintillation spectrometer. The silica gel does not cause significant quenching. The activity of the cholesterol 7α-hydroxylase enzyme was then expressed as percentage conversion of added substrate to 7α-hydroxycholesterol.

Radioactive thin layer scans of the pattern of products obtained from incubations with and without the inclusion of 10 mM β-mercaptoethylamine are shown in Fig. 7. This thiol is effective in preventing the formation of "autoxidation products" of cholesterol by liver microsomes (Section 1).

H. The Determination of 7α-Hydroxycholesterol

The development of this procedure for determining 7α-hydroxycholesterol is discussed in detail in Section 3. The method used is outlined in Fig. 8.

Incubations of liver microsomes were conducted as already described but without the addition of (4-^{14}C) cholesterol. After stopping the reactions with methanol, (^{3}H) 7α-hydroxycholesterol (0·04 μC) was added to each flask. Lipids were extracted and subjected to TLC as before, but standard 7α-hydroxycholesterol was run at the edge of each plate. This was located by spraying with phosphomolybdic acid. A bright turquoise colour is

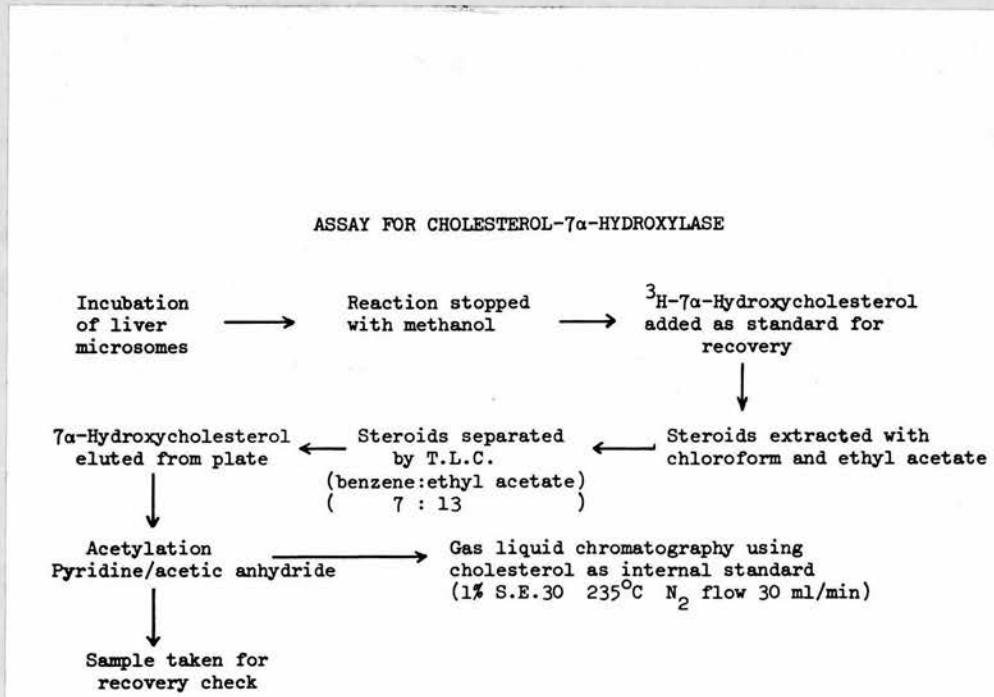

ASSAY FOR CHOLESTEROL-7α-HYDROXYLASE
Incubation of liver microsomes
Reaction stopped with methanol
^{3}H-7α-Hydroxycholesterol added as standard for recovery
Steroids extracted with chloroform and ethyl acetate
Steroids separated by T.L.C.
(benzene:ethyl acetate)
(7 : 13)
7α-Hydroxycholesterol eluted from plate
Acetylation
Pyridine/acetic anhydride
Gas liquid chromatography using cholesterol as internal standard
(1% S.E.30 235°C N_2 flow 30 ml/min)
Sample taken for recovery check

Figure 8

obtained almost immediately at room temperature. The area of the plate corresponding to 7α-hydroxycholesterol was removed (without removing the standard) into a centrifuge tube and the silica gel was extracted twice with dichloromethane. The extracts were combined and concentrated and the 7α-hydroxycholesterol was acetylated essentially according to the method of Norymberski and Riondel (1967). Each sample was applied in ether on to a small strip of filter paper (Watmans No. 1) 3 cm by 0'8 cm which was then suspended from a glass hook in a sealed vessel above a mixture of dry pyridine and acetic anhydride (2:1 v/v). This vessel could incorporate eight filter papers at one time. The samples were left to acetylate at room temperature for at least 16 hours. This procedure results in complete conversion to the diacetate. The filter papers were dried in air and eluted with 10 mls of dichloromethane. 1 ml was removed into a scintillation vial, evaporated and counted in the Packard liquid scintillation spectrometer. This gave an estimation of the efficiency of the recovery of the procedure. The remainder was concentrated and a known quantity of cholesterol was added as an internal standard for gas liquid chromatography (GLC). A sample of this mixture was injected on to the GLC column in acetone. (The conditions for chromatography are described below.) The cholest-5-ene-3β,7α-diol diacetate in the sample was quantified by comparing the ratio of the heights of the peaks obtained for the diacetate and cholesterol with a standard curve of actual ratio versus peak height ratio. This value was corrected for recovery losses and hence the mass of 7α-

hydroxycholesterol formed during the incubation was calculated. The enzyme activity was expressed as moles of 7α -hydroxy-cholesterol formed per minute per mg of protein. Microsomal protein was measured by the biuret method.

I. Conditions for Gas Liquid Chromatography

All gas chromatographic work was carried out on a Pye 104 gas chromatogram equipped with a flame ionisation detector. Columns were 1.75 metres long of 4 mm i.d. glass tubing silanised with dichlorodimethylsilane. The liquid phase used was 1% SE 30 on 100-120 mesh Gas-Chrome Q (Applied Science Laboratories Inc.). The column oven temperature was 235°C, the injection port temperature was 300°C and the detector oven temperature was 240°C. Nitrogen was used as carrier gas at a flow rate of 30 ml per minute.

J. The Determination of Cholesterol

Microsomal cholesterol was measured by gas liquid chromatography. (4-^{14}C) cholesterol (60 mC/mMole) was added to the microsomal sample as an internal standard for recovery. Lipid was extracted with chloroform-methanol (1:1 v/v) and chromatographed by TLC in a solvent system consisting of di-isopropylether: petroleum spirit: :70:30. The area corresponding to cholesterol was located using the Panax thin layer scanner and removed into a centrifuge tube. Cholesterol was extracted from the silica gel using chloroform-methanol and a sample was removed for liquid scintillation counting. A sample of the remainder was applied to a GLC column using pregnenolone acetate as an internal standard. The conditions used are described above. The mass of

cholesterol was calculated by measuring the ratio of the peak heights and comparing with a standard curve as already described for 7α -hydroxycholesterol. This value was corrected for losses on the basis of the calculated recovery of the ^{14}C standard.

Total liver cholesterol was measured by the Liebermann-Burchardt colour reaction.

K. Assay for Aminopyrine Demethylase

Aminopyrine is N-demethylated by liver microsomes in the presence of NADPH and molecular oxygen with the production of formaldehyde (Ernster and Orrenius, 1966).

The incubation medium used was the same as that for the cholesterol 7α -hydroxylase assay, but omitting radioactive cholesterol and β-mercaptoethylamine and incorporating 10 mM aminopyrine. The reaction was conducted in air at 37°C for 40 minutes, and stopped with 0'5 ml of 60% perchloric acid. The formaldehyde produced during the incubation was assayed according to method of Nash (1953). The reaction mixture was centrifuged to remove denatured protein and the supernatant was incubated with an equal volume of the formaldehyde reagent (2 M ammonium acetate, 0'05 M acetic acid, 0'02 M acetyl acetone) at 37°C for 40 minutes. The optical density of the resulting yellow colour was read in a Unicam SP 600 spectrophotometer at 412 nm and the concentration of formaldehyde calculated using an extinction coefficient of 8×10^{-3}M. The enzyme activity was expressed as moles of formaldehyde produced per minute per mg of microsomal protein.

L. Assay for Cholesterol Biosynthesis

Animals were anaesthetised with ether and the livers were rapidly removed, minced and homogenised in three volumes of Bucher's medium (Bucher et al, 1959; Table 1). The homogenate was centrifuged at 18,000 g for 15 minutes to remove nuclei, cell debris, unbroken cells and the mitochondrial fraction and the resulting supernatant was used in the incubations. The incubation medium is shown in Table 2 and the reaction was carried out at 37°C for 3½ hours. The reaction was stopped with 10 mls of 10% KOH in methanol (w/v), a sample of (7α-^{3}H) cholesterol was added as a recovery indicator and the mixture was refluxed under nitrogen. Lipid was extracted with chloroform and boiling ethyl acetate, the extracts were combined, washed with sodium bicarbonate solution, concentrated and subjected to TLC in a solvent system of di-isopropylether:petroleum spirit: :70:30. The area of the plate corresponding to cholesterol was removed into a scintillation vial and counted as described previously. The incorporation of ^{14}C from (1-^{14}C) acetate into cholesterol was corrected for losses as measured by the recovery of (7α-^{3}H) cholesterol. The rate of cholesterol biosynthesis from acetate was expressed as counts per minute of ^{14}C incorporated per incubation.

M. Determination of Cytochrome P450

Cytochrome P450 estimations were carried out essentially according to the method of Omura and Sato (1964) in an Aminco-Chance spectrophotometer with the instrument in the dual wavelength mode set to measure changes in the

Table 1

Bucher's Medium

0.004 M $MgCl_2$

0.03 M nicotinamide

0.125 M sucrose

0.001 M EDTA

0.1 M potassium phosphate buffer, pH 7.4

Table 2

Incubation medium for cholesterol biosynthesis in vitro

2 ml Bucher's medium

1 µmole NADP

5 µmoles Glucose-6-phosphate

0.2 I.U. Glucose-6-phosphate dehydrogenase

5 µmoles NAD

20 µmoles Glutathione

2 µmoles ATP

5 mM sodium acetate

10 µC sodium (1-^{14}C) acetate (29 mC/mM)

2 ml liver 18000 g supernatant

optical density at 450 nm relative to 490 nm. Liver microsomes were suspended in 0'1 M phosphate buffer pH 7'4 at a concentration of 1-3 mg protein per ml, a few grains of sodium dithionite were added to reduce the cytochromes and a zero reading was made in the spectrophotometer. Carbon monoxide gas was then bubbled through the cuvette for 20 seconds and a new reading was made. The amount of cytochrome P450 was calculated using an extinction coefficient of 91 x $10^{-3}M^{-1}$ (Omura and Sato, 1964) and was expressed as nmoles per mg of microsomal protein.

N. Assay of NADPH Cytochrome c Reductase Activity

The rate of this reaction was measured using the Aminco-Chance spectrophotometer in the dual wavelength mode. The increase in optical density at 551 nm relative to 540 nm was measured. The cuvette contained 2'5 ml 0'1 M phosphate buffer pH 7'4. 0'2 ml NADPH generator (1 μ mole NADP, 10 μ mole glucose 6-phosphate, 0'2 I.U. glucose 6-phosphate dehydrogenase) and 0'05 ml of microsomal suspension and the reaction was allowed to proceed for 2-3 minutes. The rate of the reaction was calculated using the initial slope of the line obtained and an extinction coefficient for reduced cytochrome c at 551 nm of 19 x $10^{-3}M^{-1}$. The rate was expressed as nmoles of cytochrome c reduced per minute per mg protein.

O. Measurement of Difference Spectra

Microsomal difference spectra were obtained using the Aminco-Chance spectrophotometer set in the split-beam mode. Microsomal suspensions containing 2-3 mg protein per ml of phosphate buffer pH 7'4 were divided between two cuvettes.

The spectrum was scanned from 370 to 450 nm with and without the addition of a solution of the compound under study to the sample cuvette, the same volume of solvent being added to the reference cuvette. Increasing the concentration of the compound results in an increase in the difference in optical density between the minimum and maximum wavelengths of the difference spectrum until saturation is achieved. The binding constant (Ks) for the compound is the concentration at which this difference in optical density is half-maximal.

P. Preparation of Androst-5-ene-3β-ol and Pregn-5-ene-3β-ol

These compounds, the ketone reduction products of dehydroepiandrosterone and pregnenolone respectively, were prepared using the Huang-Minlon modification of the Wolf-Kishner method for the reduction of ketones (Huang-Minlon 1949).

1 g of steroid, 2 g potassium hydroxide, 0'5 mls of hydrazine hydrate and 50 mls triethylene glycol were refluxed together for approximately 0.5 hours to form the hydrazone. Water and excess hydrazine were removed by heating without the reflux condenser until the temperature rose to 195-200°C and refluxing was continued for a further 2 hours to decompose the hydrazone. The cooled solution was diluted with 10 mls of water and extracted four times with ether. The ether extract was washed once with water and evaporated to dryness.

Androst-5-ene-3β-ol was recrystallised from ethyl acetate to give white needles.

m.p. 133-135°C (literature value 132-133°C)

TLC in ethyl acetate: benzene: :5:1: One spot R_F 0'82

Pregn-5-ene-3β-ol was recrystallised from methanol. m.p. 134-136°C (literature value 133-134°C)

TLC in ethyl acetate:benzene: :5:1: One spot R_F 0'84.

Mass spectra were obtained for these compounds using a Hitachi Perkin-Elmer RMU 6E mass spectrometer. Molecular weights for androst-5-ene-3β-ol and pregn-5-ene-3β-ol were confirmed as 274 and 302 respectively.

Q. The Preparation of Tritiated Androst-5-ene-3β-ol, Pregn-5-ene-3β-ol and Cholest-5-ene-3β,7α-diol

These steroids were tritiated according to the method of Wilzbach (1957). A sample of the compound (5 mg) was spread in a thin film around a flask which was exposed to tritium vapour at low pressure for four days. The steroid was repeatedly washed with methanol to remove free tritium and then purified by TLC (ethyl acetate:benzene: :5:1) to remove polar degradation products.

Steroid	Specific Activity
androst-5-ene-3β-ol	2'0 x 10^7 d.p.m. per mg
pregn-5-ene-3β-ol	1'0 x 10^8 d.p.m. per mg
cholest-5-ene-3β,7α-diol	2'0 x 10^7 d.p.m. per mg

R. Assay for the Microsomal Oxidation of Dehydroepiandrosterone, Pregnenolone, Androst-5-ene-3β-ol and Pregn-5-ene-3β-ol

The assay medium was the same as that used for cholesterol oxidation, but omitting β-mercaptoethylamine and (4-^{14}C) cholesterol, and incorporating the radioactive steroid whose oxidation was being studied. The steroid was diluted to the required specific activity and 0'1 μC was

added in acetone to the incubation medium. (4-^{14}C) dehydroepiandrosterone and (4-^{14}C) pregnenolone (60 mC/mM) were obtained from the Radiochemical Centre, Amersham. (^{3}H) androst-5-ene-3β-ol and (^{3}H) pregn-5-ene-3β-ol were prepared as already described. The oxidation of these compounds by liver microsomes is discussed in Section 6. The reactions were stopped with methanol and lipid was extracted exactly as described for cholesterol 7α-hydroxylase (Fig.6). The radioactive steroids were separated by TLC using a solvent system of ethyl acetate:benzene: :5:1 and counted by liquid scintillation spectrometry. The percentage conversion of substrate to product was calculated and the enzyme activity expressed as moles of product formed per minute per mg of microsomal protein.

S. Reagents and Materials

All reagents were of Analar grade and were supplied by B.D.H. or Sigma unless otherwise stated. Glucose 6-phosphate and NADP were supplied by Boehringer. Acetone was redistilled after refluxing with potassium permanganate. Methanol and ethanol were refluxed with potassium hydroxide before redistilling. Dichloromethane was also redistilled before use. The toluene for liquid scintillation counting was washed with concentrated sulphuric acid, then with water and finally dried over sodium sulphate. (4-^{14}C) cholesterol obtained from the Radiochemical Centre, Amersham, was purified by TLC on Keiselgel H (Merck) using a solvent system of di-isopropylether:petroleum spirit: :70:30 prior to use.

SECTION 3

AN ABSOLUTE ASSAY FOR CHOLESTEROL 7α -HYDROXYLASE

A. Introduction

Colorimetric methods for determining 7α -hydroxycholesterol are not sufficiently sensitive to measure the amounts of this steroid which can be formed during an incubation of liver microsomes. For example, the Lifschütz reaction (Bergström and Wintersteiner, 1942) is suitable for measuring quantities of the order of 100 μg. Thus the method which has been used in this and in other laboratories has involved the addition of exogenous radioactive cholesterol to liver subfractions and measuring the percentage of radioactivity incorporated into the product, 7α -hydroxycholesterol (Mitton et al, 1971; Danielsson et al, 1967; Shefer et al 1968). Using this method to compare the enzyme activity in the livers of different animals involves two assumptions:

(1) that the total pool of cholesterol in the liver subfractions is the same,

(2) that the added exogenous tracer cholesterol equilibrates with all the endogenous cholesterol.

Liver microsomes contain considerable amounts of endogenous cholesterol, on average approximately 500 nmoles per g of liver microsomes. This cholesterol pool is not necessarily homogeneous. Cholesterol is continually being synthesised and degraded in the liver and is also derived from intestinal absorption. Cholesterol has also a structural function in the membranes of the endoplasmic reticulum. Thus under different physiological conditions when the relative sizes of these pools may be altered, the distribution

of exogenous radioactive cholesterol between the different pools may also change. It therefore seemed desirable to establish a method for measuring directly the total mass of 7α-hydroxycholesterol formed from endogenous cold cholesterol in liver microsomal incubations. Measuring the disappearance of the substrate, cholesterol, would not be a suitable means of assaying the enzyme due to the small proportion of the endogenous cholesterol which reacts during the period for which the time course of the reaction is linear.

A method was already in use in this laboratory for measuring pregnenolone formed from cholesterol in the side chain cleavage reaction of adrenal mitochondria (Simpson et al 1971). This involves purification of the pregnenolone followed by reaction with a 3β-hydroxysteroid dehydrogenase from Pseudomonas testosteroni to form progesterone. The NADH formed during the reaction is then assayed by its fluorescence emission at 457 nm when irradiated with light at 340 nm. Although this bacterial steroid dehydrogenase is a fairly non-specific enzyme which reacts with a wide spectrum of 3β-hydroxysteroids, it was shown not to react with 7α-hydroxycholesterol.

The next method of choice was gas liquid chromatography, which was already in use in this laboratory for measuring cholesterol. However 7α-hydroxycholesterol was found to decompose on injection on to the GLC column. Under the conditions necessary to volatilise it, this compound gave at least three distinct peaks. This is in agreement with the results of Van Lier and Smith (1967), who suggest that

the major decomposition product might be similar to $\Delta^{3,5}$-cholestadiene. These workers have shown that the 3β,7α-diacetate of 7α-hydroxycholesterol does not decompose and so it was decided to attempt derivative formation combined with GLC to assay 7α-hydroxycholesterol. The most efficient method of forming the diacetate was found to be that described in Section 2. Here the derivative is formed by subjecting the diol adsorbed on filter paper to the vapours of the reactants pyridine and acetic anhydride. 16 hours at room temperature was found to give complete conversion. This method has been described by Norymberski and Riondel (1967) and is particularly convenient for GLC since there is no problem in removing the pyridine which is relatively involatile and gives a broad peak on GLC. In some experiments the diacetate was first purified by TLC in benzene: ethyl acetate: :9:1.

B. <u>The Behaviour of Cholest-5-ene-3β,7α-diol diacetate on Gas Liquid Chromatography</u>

Using the GLC conditions described in Section 2, cholest-5-ene-3β,7α-diol diacetate gave a single symmetrical peak with a retention time relative to cholesterol of 0'64. This is in contradistinction to the results of Van Lier and Smith (1967) who showed a retention time relative to cholsterol of 1'69 on SE 30 columns. It seemed possible that under our conditions the diacetate decomposes to give a product having a shorter retention time. There was no trace of any peak at longer retentions, suggesting that total decomposition occurs. To determine if the peak obtained for the diacetate in these experiments was a

decomposition product, GLC coupled to mass spectrometry was used. The peak obtained was identified as a cholestatriene. The identification of this compound is described in Appendix 1.

Assuming complete decomposition of the diacetate to a single product, it should be valid to use measurement of this decomposition product as an assay for 7α-hydroxycholesterol. This assumption is based on the fact that a single symmetrical peak was obtained and that the calibration curves such as that shown in Fig. 10 were consistent. The check of the assay procedure is shown in Fig. 11 (Part D of this section).

C. The Specificity of the Method

The separation of the lipid extract of liver microsomes by TLC should be sufficient to purify 7α-hydroxycholesterol from other products of the liver microsomal metabolism of cholesterol. Also, the conditions of the incubation are such that there should be selective formation of 7α-hydroxycholesterol. The exclusion of NAD^+ prevents the formation of 7α-hydroxycholest-4-ene-3-one, the next intermediate in bile acid biosynthesis, and the inclusion of β-mercaptoethylamine inhibits the formation of 'autoxidation products' of cholesterol (Section 1). However, the behaviour of possible 'autoxidation products' of microsomal cholesterol metabolism on the GLC column were examined in order to find out to what extent they would interfere with the estimation of 7α-hydroxycholesterol. These steroids (Mitton et al, 1971) were subjected to the same acetylation procedure as described for 7α-hydroxycholesterol.

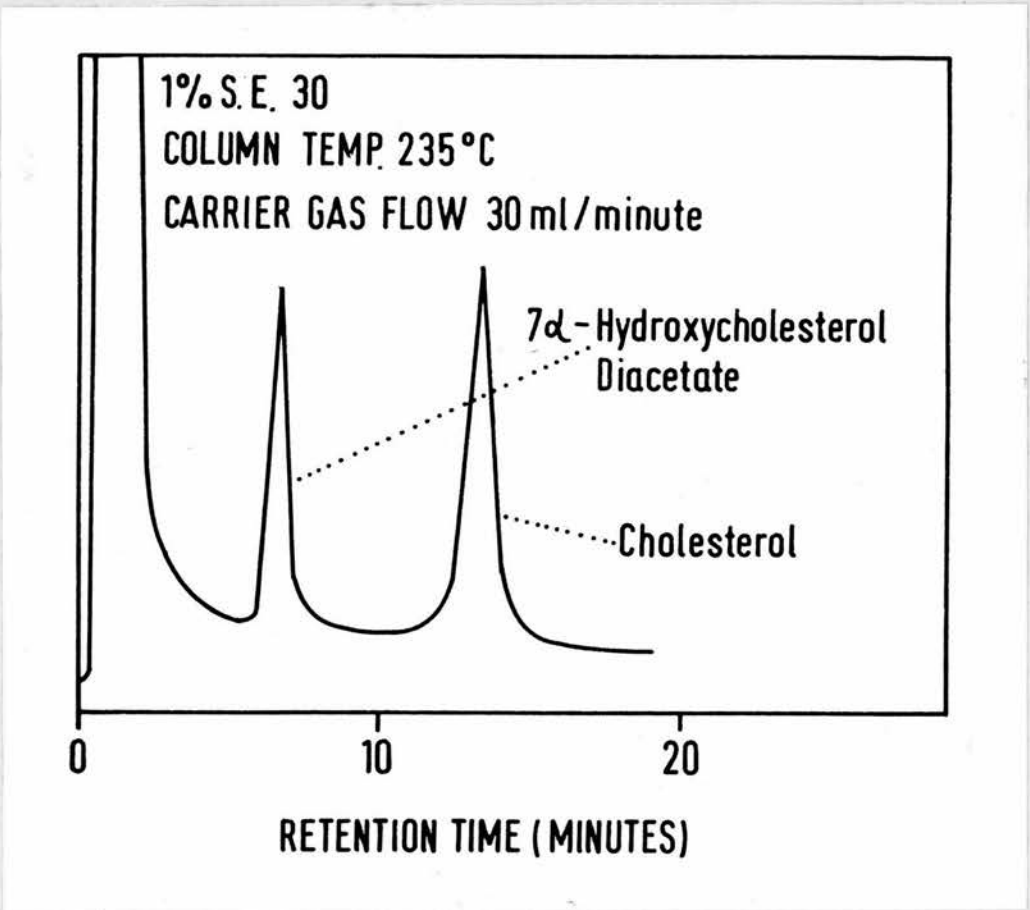


Figure 9 Gas liquid chromatographic elution pattern of cholest-5-ene-3β,7α-diol diacetate and cholesterol injected in acetone solution.

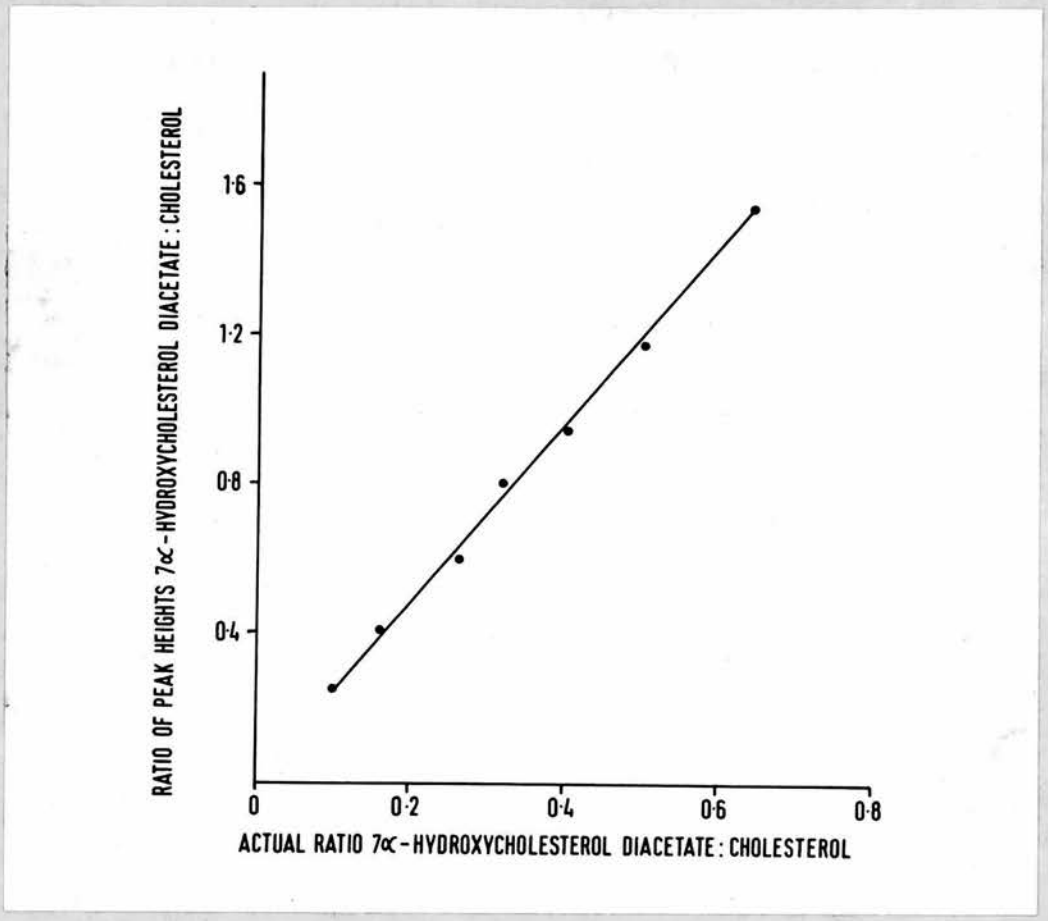


Figure 10 Calibration curve for cholest-5-ene-3β,7α-diol diacetate and cholesterol on GLC.

The retention times relative to cholesterol using the GLC conditions described in Section 2 are shown below.

Sterol Acetate	Relative Retention
cholest-5-ene-3 β,7 α -diol	0'64
cholest-5-ene-3 β,7 β -diol	0'64
3 β-hydroxycholest-5-ene-7-one	1'15
Cholestan-3β,5α,6β-triol	2'28

Thus the method does not distinguish between the epimeric diols, 7α and 7β -hydroxycholesterol, however the other 'autoxidation products' of cholesterol are well resolved.

D. Calibration of the Assay for 7α -Hydroxycholesterol

The cholest-5-ene-3 β,7α -diol diacetate in each sample was estimated by GLC using cholesterol as an internal standard, measuring the ratio of the heights of the peaks obtained and computing using a calibration curve. Fig. 9 shows the trace which is obtained on injection of a mixture of these two compounds in acetone. A typical calibration curve is shown in Fig. 10. A curve such as this was obtained for each experiment and the mass of 7α -hydroxycholesterol formed during an incubation was calculated as described in Section 2. The method was checked by adding known quantities of 7α -hydroxycholesterol to liver microsomes and comparing with observed values. Fig. 11 shows the correlation between the calculated mass of 7α -hydroxycholesterol and the actual mass.

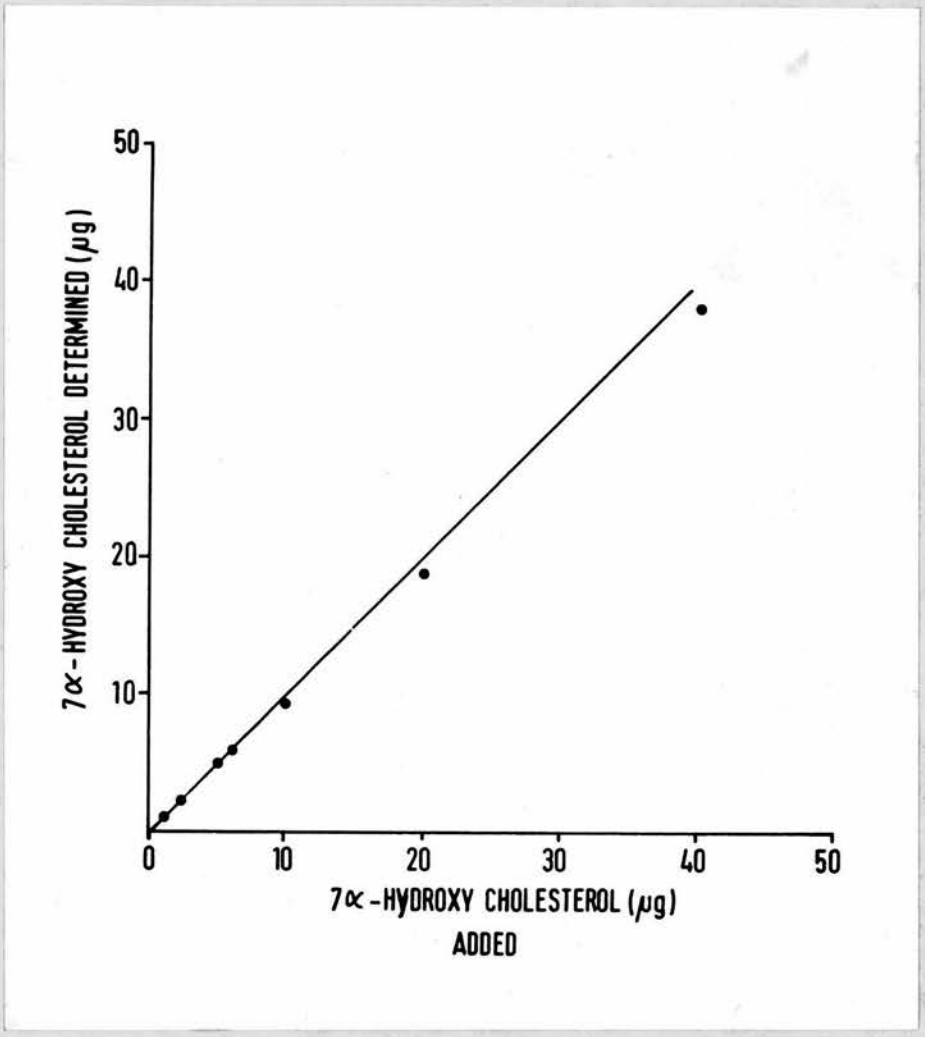


Figure 11 Check of the GLC assay for 7α-hydroxycholesterol.

E. The Sensitivity of the Method

The amount of 7α-hydroxycholesterol lost during the procedure of extraction, thin layer chromatography and acetylation can be calculated on the basis of the recovery of the standard (^{3}H) 7α-hydroxycholesterol added at the end of the incubation. The recovery obtained at each stage of the procedure is shown below for a typical experiment (mean of three samples).

Stage of Procedure	C.P.M. recovered	% recovery
Added Standard	9850	100
After extraction from microsomes	9280	94
After TLC	7570	77
After extraction from silica gel	5840	59
After acetylation	4520	46

The final recovery was found to vary between 35% and 55%. A full scale deflection of the recorder attached to the gas liquid chromatogram corresponded to 1'6 μg of cholest-5-ene-3β,7α-diol diacetate. Considering the losses during extraction, acetylation and injection on to the GLC column, the method was estimated to be sensitive to 1 μg of 7α-hydroxycholesterol. The precision of the method was determined by making several estimations on the same amount of 7α-hydroxycholesterol. Six samples of 5'0 μg of 7α-hydroxycholesterol gave an observed value of (4'9 $\pm$ 0'3) μg.

The radioactive tracer assay is sensitive to approximately 0'5% conversion of added (4-^{14}C) cholesterol. Assuming complete equilibration of tracer with endogenous substrate, this corresponds to approximately 1 μg of

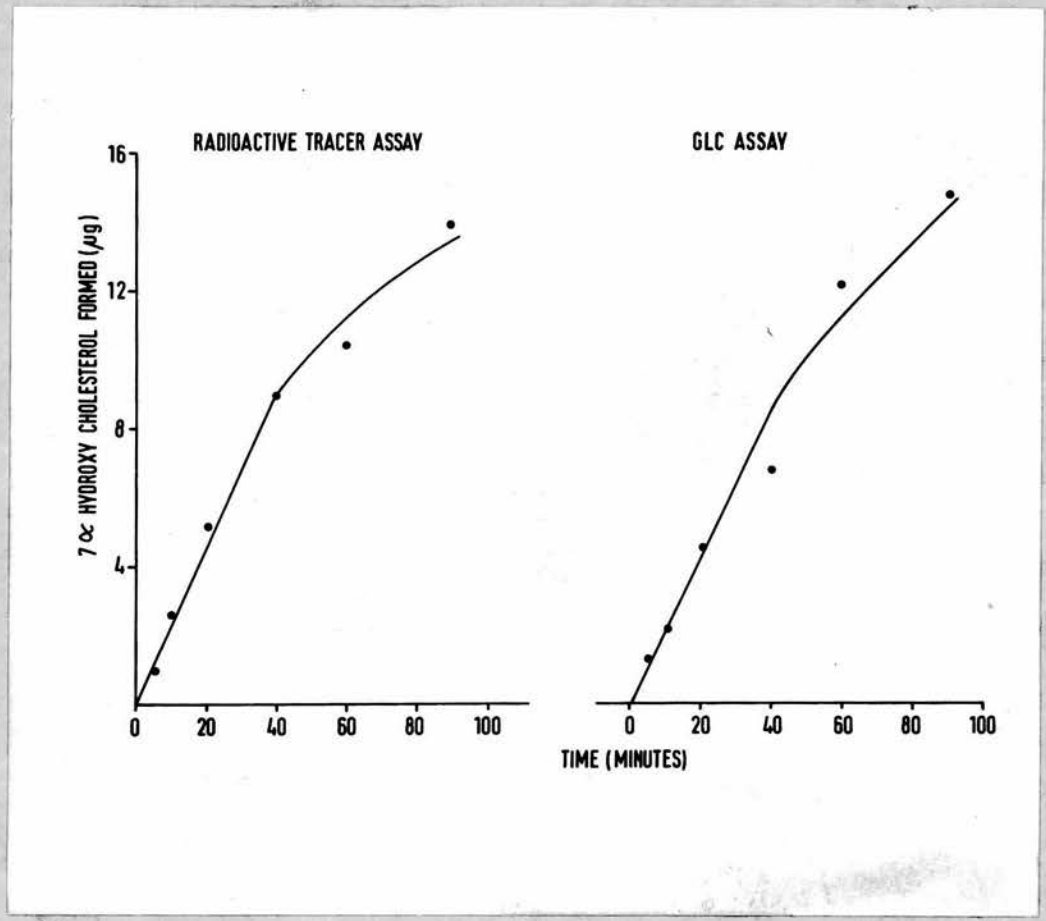


Figure 12 Time course of 7α -hydroxycholesterol formation as measured by the radioactive tracer assay and the GLC assay.

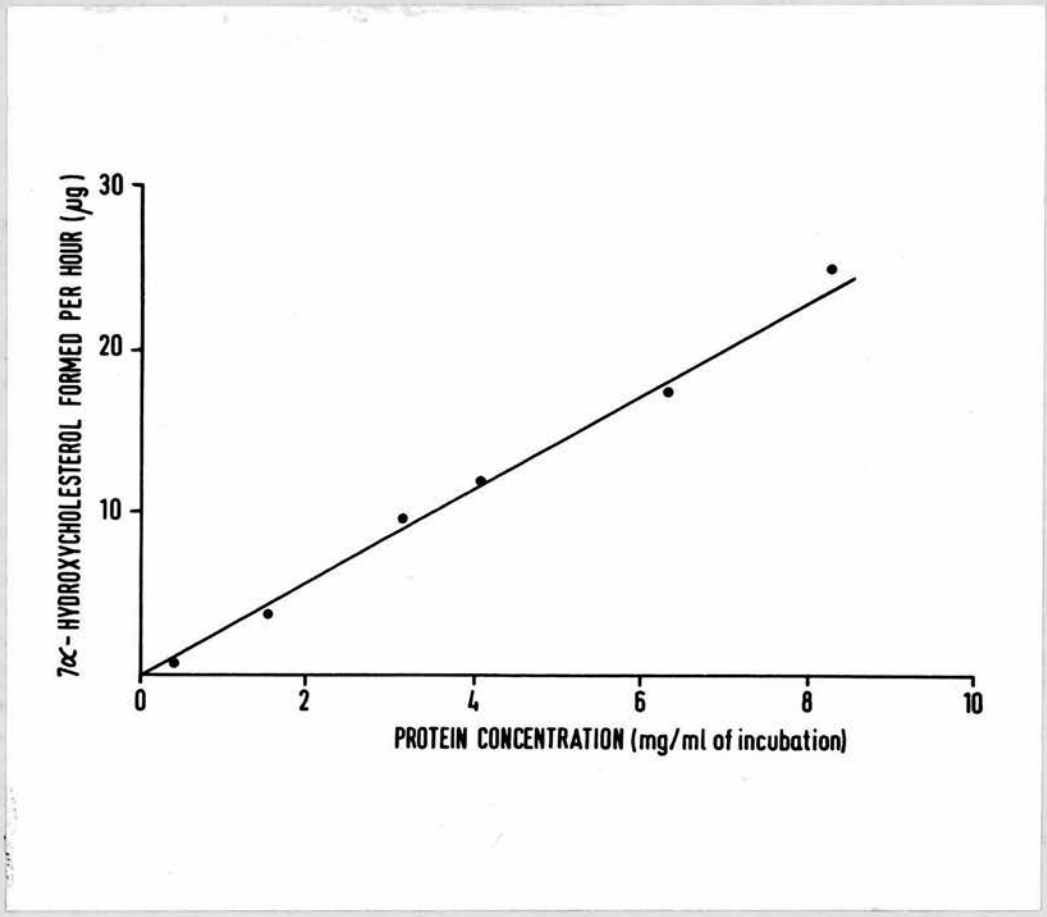


Figure 13 The effect of microsomal protein concentration on the rate of formation of 7α -hydroxycholesterol.

cholesterol, hence the GLC assay is no more sensitive than the radioactive assay. The precision of the radioactive assay was assessed by conducting identical incubations. A typical experiment in which six samples of microsomes were incubated for 1 hour gave a value of (7'18 $\pm$ 0'11)% conversion.

The GLC assay is advantageous in that it makes no assumptions regarding cholesterol pool sizes or tracer equilibration.

F. Comparison of the GLC Assay for Cholesterol 7α-Hydroxylase with the Radioactive Tracer Assay

The radioactive tracer assay for cholesterol 7α-hydroxylase assumes that exogenous (4-^{14}C) cholesterol equilibrates with all the endogenous microsomal cholesterol. If this is so, then the amount of 7α-hydroxycholesterol formed as calculated by this method should not differ significantly from the value obtained using the GLC assay. Fig. 12 shows a time course for 7α-hydroxycholesterol formation calculated using the two assays. Total microsomal cholesterol was estimated by GLC. The initial rate of formation is approximately the same in each case, suggesting that the assumption of total equilibration of tracer is valid.

G. The Effect of Microsomal Protein Concentration on 7α-Hydroxycholesterol Formation

The amount of 7α-hydroxycholesterol formed after incubating different concentrations of microsomes was measured using the GLC assay. Fig. 13 shows that the reaction is first order with respect to protein concentration.

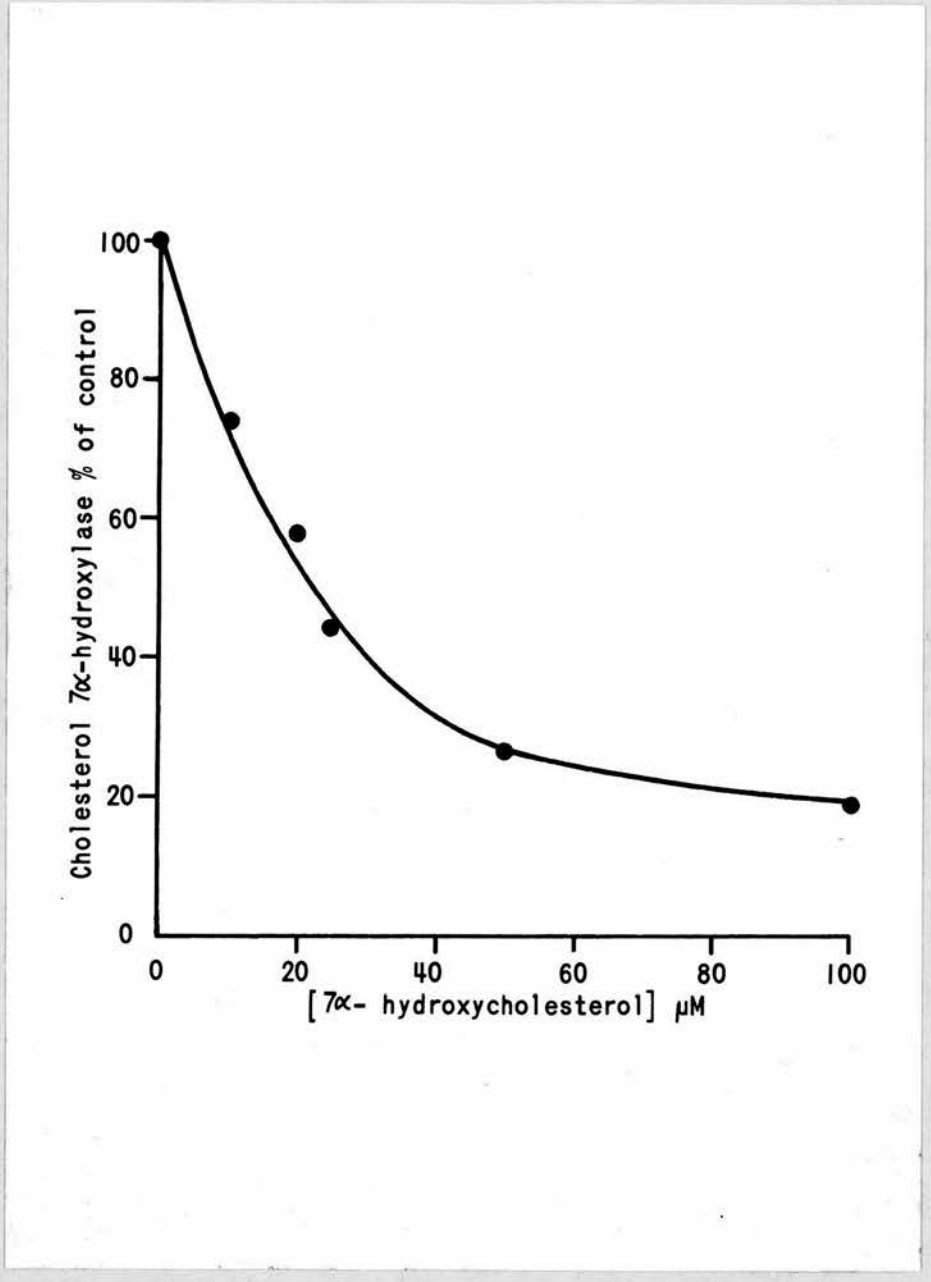


Figure 14 The effect of 7α -hydroxycholesterol on cholesterol 7α -hydroxylase *in vitro*.

H. The Effect of 7α -Hydroxycholesterol on Cholesterol 7α -Hydroxylase

The effect of the product, 7α -hydroxycholesterol, on cholesterol 7α -hydroxylase activity is shown in Fig. 14. The concentration in the initial incubation medium required for 50% inhibition is approximately 25 μM. The amount of 7α -hydroxycholesterol formed after one hour's incubation in the experiment described in Part F of this section was 12 μg. The largest value obtained in any experiment was 21 μg, which corresponds to a concentration of 7'5 μM. Thus accumulation of product is unlikely to have much effect on the reaction rate. The conditions used for assaying the enzyme are such that 7α -hydroxycholesterol accumulates in the incubation medium. In vivo, however, the presence of NAD^+ together with the active 3β -ol dehydrogenase present in liver microsomes (Hutton and Boyd, 1966; Berseus et al, 1969) results in efficient conversion to 7α -hydroxycholest-4-ene-3-one and 7α -hydroxycholesterol does not accumulate to any great extent. Thus product inhibition is unlikely to play an important physiological role in controlling enzyme activity.

SUMMARY TO SECTION 3

1) An assay procedure has been developed to determine quantitatively the mass of 7α -hydroxycholesterol produced in liver microsomal incubation.

2) The values obtained using this method agree closely with those obtained using the radioactive tracer assay, suggesting that exogenous tracer cholesterol added in acetone solution equilibrates rapidly with all the endogenous microsomal cholesterol.

3) The rate of formation of 7α-hydroxycholesterol is first order with respect to microsomal protein concentration.

4) Cholesterol 7α-hydroxylase is inhibited by the product of the reaction, 7α-hydroxycholesterol, but at concentrations higher than that which might accumulate in the incubation medium.

SECTION 4

THE CONTROL OF CHOLESTEROL 7α-HYDROXYLASE ACTIVITY IN VIVO

A. Introduction

Cholesterol 7α-hydroxylation is the initial step in the catabolism of cholesterol to bile salts and is also thought to be rate-limiting (Danielsson et al, 1967; Shefer et al, 1968, 1970; Boyd et al, 1969). Hence agents which alter the activity of this enzyme in vivo will affect the rate at which cholesterol is degraded. Since bile acid formation is quantitatively the most important pathway of cholesterol catabolism, agents which activate this pathway have been used in an attempt to lower serum cholesterol levels. For example, cholestyramine, an anion exchange resin which binds bile salts in the small intestine and so inhibits their reabsorption, stimulates bile acid production and has been used effectively to lower serum cholesterol levels in man (Bergen et al, 1959). However, in the rat cholestyramine feeding did not significantly lower serum cholesterol levels (Huff et al, 1963). In this animal hepatic cholesterol biosynthesis is markedly increased following biliary drainage (Myant and Eder, 1961) and it has since been shown that the enzyme whose activity is most affected by this procedure is HMG CoA reductase (Danielsson et al, 1967; Weis and Dietschy, 1969). Thus in the rat the steady state levels of cholesterol in the whole animal are not significantly altered by biliary diversion. The control of cholesterol metabolism in man and other species is discussed in Section 7.

Myant and Eder (1961) showed that in the biliary fistula rat the increase in cholesterol biosynthesis preceded by several hours the increase in bile acid biosynthesis. These workers suggested that bile acid formation increased as a consequence of the enhancement of cholesterol biosynthesis. It is possible that the precursor of bile acids is endogenously synthesised cholesterol and that the rate of supply of this precursor controls the rate at which bile acids are formed. Cholesterol 7α-hydroxylase activity would then depend on the availability of the substrate cholesterol. Other evidence which links cholesterol 7α-hydroxylase and cholesterol biosynthesis is the fact that cholesterol 7α-hydroxylase undergoes a diurnal variation in activity (Renson et al 1969), a property which is shared by the major rate limiting enzyme in cholesterol biosynthesis, HMG CoA reductase (Hamprecht et al 1969). Recently Alam and Glover (1972) showed that the administration of an inhibitor of cholesterol biosynthesis to bile fistula rats resulted in diminished secretion of bile acids which they suggested was a consequence of decreased supply of substrate. If this hypothesis is correct, then bile acid biosynthesis, like cholesterol biosynthesis, will be regulated by the amount of cholesterol which is absorbed from the gastrointestinal tract as suggested by Dietschy and Wilson (1970). However Cayen (1971) showed that the incorporation into the diet of tomatine, a steroid glycoside which complexes with cholesterol in the small intestine and hence prevents cholesterol absorption, significantly stimulated hepatic cholesterologenesis without simultaneously affecting bile

acid biosynthesis. This suggests that cholesterol biosynthesis and degradation can be controlled independently and that increased cholesterologenesis is not a sufficient stimulus to cause induction of bile acid biosynthesis.

Other factors which control cholesterol biosynthesis in rat liver are the amount ofcholesterol in the diet and the total caloric intake of the animal. Feeding a high cholesterol diet markedly inhibits the activity of HMG CoA reductase (Siperstein and Fagan 1966) and in the rat it stimulates the formation of bile acids (Wilson 1962). The activity of HMG CoA reductase is also reduced following starvation (Hamprecht et al 1969). The effect of bile acid absorption in controlling hepatic cholesterologenesis is under dispute. Weis and Dietschy (1969) suggested that the ability of bile acids to inhibit hepatic HMG CoA reductase in vivo could be explained in terms of their effect on increasing the intestinal absorption of cholesterol. However Hamprecht et al (1971) have challenged this by showing that cholic acid feeding inhibited hepatic HMG CoA reductase activity in rats with a lymph fistula where the amount of cholesterol reaching the liver from the intestine should be greatly reduced. It is difficult to correlate these results with Cayen's results on tomatine feeding where inhibition of cholesterol absorption without effect on bile acid absorption was accompanied by a dramatic increase in the rate of hepatic cholesterologenesis from acetate.

Cholesterol 7α-hydroxylase activity was studied under different conditions which are known to affect the rate of

hepatic cholesterol biosynthesis in order to understand more about the interrelationships between the regulation of these two systems.

B. The effect of diet on cholesterol 7α -hydroxylase

Control diets used in the animal unit of this department are a pellet diet ('Oxoid' No. 86) and a semisynthetic soft diet (described in Section 2). Prior to October 1971, animals fed on either of these diets did not differ significantly in the activity of their liver microsomal cholesterol 7α -hydroxylase (approximately 1-2% conversion of added substrate per hour). However, animals killed after this date which had been fed on pellet diet were found to have considerably greater cholesterol 7α -hydroxylase activities (4-7% conversion of added substrate per hour). To establish that this effect was due to the diet rather than to any seasonal variation in enzyme activity or change in the animals, a group of rats were maintained on the soft diet for four weeks. These were killed and their liver microsomal cholesterol 7α -hydroxylase activities compared with a group which had been maintained on the pellet diet. The results of this experiment are shown in Table 3.

Cholesterol 7α -hydroxylase activities were significantly greater in animals fed the pellet diet, suggesting that some alteration in the composition of this diet could be responsible for the effect. Consultation with 'Oxoid' on this matter failed to resolve the issue. The only suggestion they could offer was that since 1969 dieldrin had no longer been used to spray the grain used in making

Table 3

The effect of diet on rat liver cholesterol 7α-hydroxylase activity

	Soft diet	Pellet diet	Difference
Cholesterol 7α-hydroxylase % conversion to 7α-hydroxycholesterol	1.9 $\pm$ 0.3	4.4 $\pm$ 0.7	$P < 0.02$
	n = 4	n = 4	

n = no. of animals in each group.

the diet. Dieldrin is a polychlorinated insecticide which has been shown to induce the activity of the drug metabolising enzymes of rat liver (Ghazal et al 1964). It seems unlikely that removal of this compound could result in increased activity of cholesterol 7α-hydroxylase, however. As discussed in Section 5, alterations in hepatic cytochrome P450 levels do not seem to reflect changes in cholesterol 7α-hydroxylase activity.

Bile acid metabolism is known to be affected by the integrity of the enterohepatic circulation; agents which bind bile salts in the small intestine cause increased secretion of bile salts from the liver. Normal constituents of any diet probably adsorb bile salts to a certain extent. Serafin and Nesheim (1970) showed that undigested protein material in the gastrointestinal tract of chickens fed soyabean meal bound bile salts and increased the rate of turnover of bile acids. Eastwood and Hamilton (1968) showed that a constituent of vegetable fibrous tissue bound bile acids and salts. They established that the fraction responsible was lignin, a structural aromatic polymer having functional groups which may or may not be methylated. The adsorption was abolished in the presence of 6M urea and postulated to be hydrophobic in nature. 'Oxoid' No. 86 pellets contain approximately 3% by weight of crude fibres which come from grain and probably contain lignin. It is possible that the structure of the lignin in these fibres might vary according to the conditions under which the grain is grown and hence alter the capacity to adsorb bile salts. This would in turn alter the activity of the cholesterol 7α-hydroxylase.

Another factor which might affect bile acid metabolism is the intestinal flora (Section 1). Dietary effects might be secondary to changes in bacterial action. N.B. Anti biotics have never at any time been incorporated into 'Oxoid' diets.

Whatever the explanation for the increase in enzyme activity, it is evident that dietary composition plays a major part in the regulation of bile acid metabolism. In all subsequent experiments animals were fed the soft diet for at least four weeks prior to treatment.

C. The effect of Cholestyramine Feeding on Cholesterol 7α-Hydroxylase

Cholestyramine is an anionic exchange resin which binds bile salts in the gut. The effect of this agent on liver weight, microsomal protein content and cholesterol 7α-hydroxylase activity as measured by both assays is shown in Table 4.

The treatment significantly increased the activity of cholesterol 7α-hydroxylase by three to four-fold as measured by both methods. Liver weight and microsomal protein content were not affected.

D. The effect of Cholesterol Feeding

Feeding a high cholesterol diet is known to increase the total cholesterol level in rat liver, the excess being mainly in the form of cholesterol esters (Gould 1955). Fig. 15 confirms this effect on the animals used in these experiments. The experimental group received a diet containing 1% (w/w) of cholesterol. Total cholesterol

Table 4

The effect of cholestyramine treatment on rat liver cholesterol 7α-hydroxylase activity

		Control	Cholestyramine	Difference
	Liver weight(g)	8.0 ± 1.0	9.0 ± 1.0	N.S.
	Microsomal protein (mg/g liver)	12.9 ± 1.5	11.4 ± 2.0	N.S.
Cholesterol 7α-hydroxylase	% conversion to 7 α-hydroxy-cholesterol	2.9 ± 1.4	9.5 ± 2.5	P < 0.01
	pmoles/minute/ mg protein	14.0 ± 1.5	39.9 ± 9.0	P < 0.01
		n = 8	n = 8	

N.S. not significant.

n = no. of animals in each group.

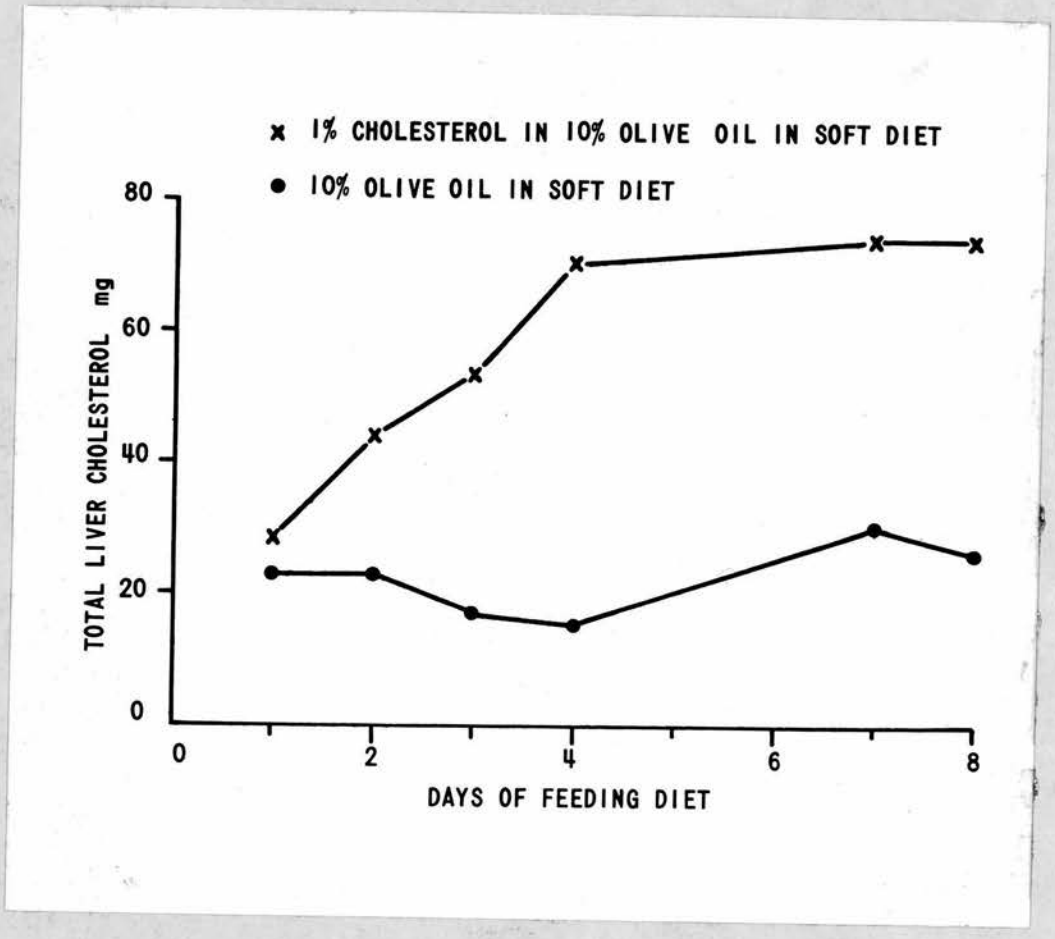


Figure 15 The effect of cholesterol feeding on total liver cholesterol content.

was measured by the Liebermann-Burchardt reaction. The hepatic cholesterol level increased to approximately three times the control and appeared to stabilise after 4-5 days. In the rat a high cholesterol diet leads to inhibition of hepatic cholesterologenesis and increased bile acid production (Siperstein and Fagan 1963; Wilson 1962). The effect of feeding a high cholesterol diet on cholesterol 7α-hydroxylase activity is shown in Table 5. The specific activity of the enzyme was measured by the GLC method. These results show that cholesterol feeding significantly increased the specific activity of the enzyme. Total microsomal cholesterol levels were also increased by about 30% as measured by GLC. Liver weight and microsomal protein content were not affected.

E. The effect of starvation on Cholesterol 7α-Hydroxylase

The starved animals in this experiment were kept without food for 48 hours. Table 6 shows the effect of this treatment on liver weight, microsomal protein content and liver microsomal cholesterol 7α-hydroxylase. These were all significantly reduced by starvation. The effect on liver weight and microsomal protein content means that the decreased production of 7α-hydroxycholesterol per liver is even more marked than is suggested by the decrease in specific activity of the enzyme.

F. The effect of biliary drainage in the starved rat

Starvation markedly inhibits the activity of hepatic HMG CoA reductase, the major rate limiting enzyme in cholesterol biosynthesis. Starved animals were subjected to biliary drainage in order to determine whether cholesterol

Table 5

The effect of cholesterol feeding on rat liver cholesterol 7α -hydroxylase activity

	Control	Cholesterol-fed	Difference
Liver weight (g)	8.5 $\pm$ 1.2	9.3 $\pm$ 0.8	N.S.
Microsomal protein (mg/g liver)	14.2 $\pm$ 2.1	14.3 $\pm$ 1.8	N.S.
Cholesterol 7α - hydroxylase (pmoles/ minute/mg protein)	12.4 $\pm$ 1.7	18.0 $\pm$ 0.8	$P < 0.05$
Microsomal cholesterol (μg/g liver)	208 $\pm$ 29	268 $\pm$ 43	$P < 0.01$
	n = 8	n = 8	

N.S. not significant

n = no. of animals in each group.

Table 6

The effect of starvation for 48 hours on rat liver cholesterol 7α-hydroxylase activity

	Control	Starved	Difference
Liver weight (g)	9.9 ± 0.5	7.3 ± 1.2	$P < 0.01$
Microsomal protein (mg/g liver)	11.2 ± 1.0	7.4 ± 0.7	$P < 0.001$
Cholesterol 7α-hydroxylase (pmoles/minute/mg protein)	16.6 ± 2.2	5.8 ± 0.7	$P < 0.001$
	n = 4	n = 4	

n = no. of animals in each group.

Table 7

The effect of biliary drainage in the starved rat. The animals were starved for 72 hours and bile duct-cannulated for 48 hours prior to death.

	Control	Biliary drained	Difference
Liver weight (g)	4.5 ± 1.0	6.0 ± 1.0	N.S.
Microsomal protein (mg/g liver)	11.4 ± 2.3	12.8 ± 3.2	N.S.
Cholesterol 7α-hydroxylase (pmoles/minute/mg protein)	5.6 ± 2.1	16.1 ± 4.8	$P < 0.05$
	n = 6	n = 6	

N.S. not significant

n = no. of animals in each group.

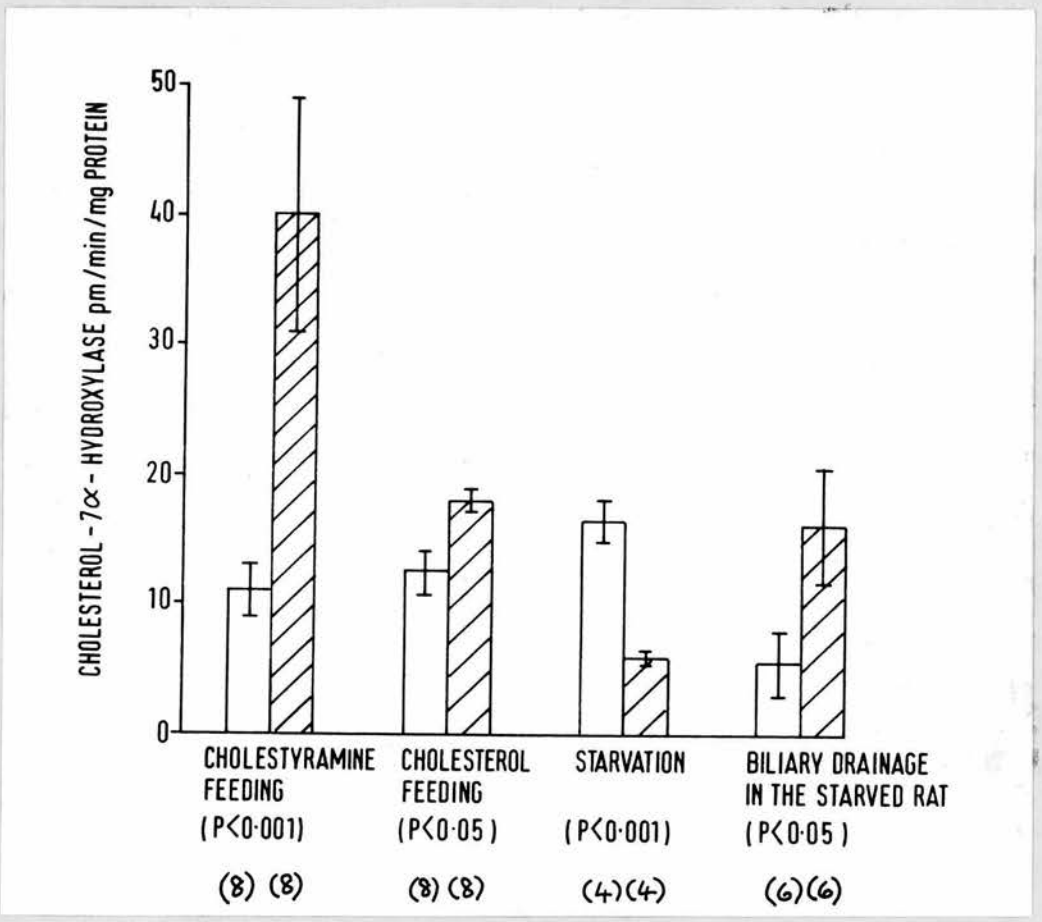


Figure 16 The effect of different *in vivo* treatments on cholesterol 7α-hydroxylase activity *in vitro*. Hatched histograms are experimental, blank are controls. The number of animals in each group is shown in parentheses.

7α-hydroxylase activity could be induced under conditions in which cholesterol synthesis is inhibited. Animals were kept without food throughout the experiment. After 24 hours one group was subjected to bile duct cannulation and another group was sham operated. After 72 hours the animals were killed and liver microsomal cholesterol 7α-hydroxylase activities measured. The results are shown in Table 7. Biliary drainage significantly increased cholesterol 7α-hydroxylase activity in the starved rat.

The effects of cholestyramine feeding, cholesterol feeding, starvation and biliary drainage plus starvation are summarised in Fig. 16.

G. <u>The effect of Tomatine Feeding on Cholesterol Biosynthesis and Cholesterol 7α-Hydroxylase</u>

Cayen (1971) showed that the incorporation into the diet of tomatine, a steroid glycoside which complexes with cholesterol and when fed prevents cholesterol absorption, significantly increases the rate of hepatic cholesterol biosynthesis without simultaneously affecting bile acid biosynthesis. This suggests that these two pathways are controlled independently. The effect of tomatine on rat liver cholesterol 7α-hydroxylase was investigated. The agent was administered at 1% in the diet for one week and cholesterol 7α-hydroxylase and cholesterol biosynthesis from acetate were measured. The results were compared with the effect of cholestyramine on these systems and are shown in Table 8.

Tomatine feeding significantly increased cholesterol biosynthesis from acetate but had no effect on cholesterol

Table 8 The effect of cholestyramine and tomatine feeding on cholesterol biosynthesis from (1-^{14}C) acetate, cholesterol 7α-hydroxylase and microsomal cholesterol levels in rat liver

	CHOLESTYRAMINE			TOMATINE		
	Control	Treated	Difference	Control	Treated	Difference
Cholesterol synthesis (c.p.m. incorporated from (1-^{14}C) acetate into (^{14}C) cholesterol per incubation)	761 $\pm$ 194 (n = 4)	3438 $\pm$ 366 (n = 4)	$P < 0.001$	1299 $\pm$ 262 (n = 14)	4489 $\pm$ 858 (n = 15)	$P < 0.005$
Cholesterol 7α-hydroxylase (% conversion to 7α-hydroxycholesterol)	3.4 $\pm$ 0.5 (n = 4)	11.6 $\pm$ 0.5 (n = 4)	$P < 0.001$	2.9 $\pm$ 0.7 (n = 14)	2.8 $\pm$ 1.0 (n = 15)	N.S.
Microsomal cholesterol (µg/g liver)	243 $\pm$ 48 (n = 8)	218 $\pm$ 35 (n = 8)	N.S.	249 $\pm$ 43 (n = 8)	256 $\pm$ 33 (n = 8)	N.S.

N.S. not significant

n = no. of animals in each group.

7α-hydroxylase, whereas cholestyramine activated both of these systems. Tomatine seems to have a similar effect to digitonin in complexing with cholesterol; however it is non-toxic in that the complex is not adsorbed. The animals showed normal weight gain and there was no effect on total liver weight or microsomal protein content. Neither tomatine nor cholestyramine had any effect on microsomal cholesterol levels. In the former case increased cholesterol biosynthesis is compensated for by increased excretion of neutral steroids and in the case of the latter by increased bile acid production. The results show that increased cholesterol synthesis *per se* is not sufficient to stimulate the formation of 7α-hydroxycholesterol.

Summary to Section 4

(1) Rat liver cholesterol 7α-hydroxylase activity was influenced by the type of diet on which the animals were maintained. Rats fed on the pellet diet had significantly greater enzyme activities than those fed on the semi-synthetic soft diet.

(2) Cholesterol feeding significantly increased the activity of rat liver cholesterol 7α-hydroxylase.

(3) Starvation significantly reduced cholesterol 7α-hydroxylase activity.

(4) When starved animals were subjected to biliary drainage liver microsomal cholesterol 7α-hydroxylase activity was significantly increased over controls.

(5) Tomatine feeding significantly increased hepatic cholesterol biosynthesis from acetate without affecting cholesterol 7α-hydroxylase, whereas cholestyramine feeding activated both of these systems.

SECTION 5

CHOLESTEROL 7α-HYDROXYLASE AS A LIVER MICROSOMAL MIXED FUNCTION OXIDASE

A. Introduction

Rat liver microsomal cholesterol 7α-hydroxylase is a mixed function oxidase requiring molecular oxygen and NADPH. The terminal oxidase for this reaction is cytochrome P450, as shown by the inhibition by carbon monoxide which is reversed by monochromatic light at 450 nm (Boyd *et al* 1971). Cytochrome P450 is reduced by NADPH, this reduction being mediated by a flavoprotein. Other compounds with a suitable redox potential can accept electrons from the reduced flavoprotein, for example cytochrome c, and in fact this flavoprotein was discovered as an NADPH cytochrome c reductase (Horecker 1950). There is considerable evidence that this flavoprotein participates in the reduction of cytochrome P450 in mixed function oxidations. These cytochrome P450-dependent hydroxylations are inhibited by cytochrome c, which presumably competes with cytochrome P450 for electrons from the reduced flavoprotein (Oshino *et al* 1968). Certain microsomal hydroxylase activities can be reconstituted by partially purified preparations of cytochrome P450 and flavoprotein (Lu and Coon 1968). An antibody prepared against the flavoprotein, NADPH cytochrome c reductase, was shown to inhibit cytochrome P450-dependent mixed function oxidase activity (Masters *et al* 1971). There is at present no evidence for the participation of any other electron transferring component in the reduction of cytochrome P450 in liver microsomes. In some tissues, for example adrenal

cortex mitochondria, the transfer of electrons from the flavoprotein to cytochrome P450 is mediated by a non-haem iron protein; however there is no evidence for any involvement of non-haem iron in the reduction of liver microsomal cytochrome P450 by NADPH.

The liver microsomal mixed function oxidase system catalyses the oxidation of drugs, carcinogens and steroids and can be induced by the administration of certain oxidisable substrates. This induction mechanism appears to be non-specific as phenobarbitone treatment results in an increase in the activity of the enzymes which metabolise not only phenobarbitone but also a large number of other substrates (review by Conney 1967). The induction of enzyme activity is accompanied by an increase in the content of cytochrome P450 and also the activity of NADPH cytochrome c reductase, suggesting that these components might be rate-limiting in the overall hydroxylation mechanism (Orrenius *et al* 1965). Present evidence suggests that there are two forms of cytochrome P450, one form with the haem iron in the high spin state which is induced by 3-methylcholanthrene treatment, and a low spin form which is induced by phenobarbitone (Hildebrandt *et al* 1968). The formation of difference spectra on the addition of an oxidisable substrate to oxidised microsomal suspensions (Remmer *et al* 1966; Schenkman *et al* 1967a) suggests that cytochrome P450 is the substrate binding site, and studies by Lu *et al* (1972) suggest that the difference in specificity of the hydroxylases induced by phenobarbitone and 3-methylcholanthrene is due to the haemoprotein moiety

Cholesterol 7α-hydroxylase appears to differ from the other liver microsomal cytochrome P450-dependent mixed function oxidases in that the enzyme induction which occurs following biliary diversion is not accompanied by a simultaneous increase in the liver content of cytochrome P450 (Boyd *et al* 1969), suggesting that this cytochrome is not rate-limiting in cholesterol hydroxylation. The effect of phenobarbitone treatment on cholesterol 7α-hydroxylase is under dispute. Einarsson and Johansson (1968b) and Boyd *et al* (1969) could show no significant effect, whereas Shefer *et al* (1968) reported a several-fold stimulation. However a recent report suggests that this discrepancy may be due to the difference in the strain of rats used (Shefer *et al* 1972).

This section concerns experiments which investigate the relationship of cholesterol 7α-hydroxylase to the rat liver microsomal mixed function oxidase system which hydroxylates drugs. In some experiments the enzyme aminopyrine demethylase has been studied for comparative purposes.

B. Cholestyramine Treatment

The effect of cholestyramine treatment on cholesterol 7α-hydroxylase, NADPH cytochrome c reductase, cytochrome P450 and aminopyrine demethylase is shown in Table 9. Although cholesterol 7α-hydroxylase activity was significantly increased by this treatment, there was no effect on aminopyrine demethylase activity or on the components of the electron transport system cytochrome P450 or NADPH cytochrome c reductase.

Table 9 Effect of Cholestyramine feeding on liver mixed function oxidase system

	Cholesterol-7α-hydroxylase pmoles/min/mg protein	Cytochrome P450 nmoles/mg protein	NADPH cytochrome C reductase nmoles/min/mg protein	Aminopyrine Demethylase nmoles/min/mg protein
CONTROL	14.0 ± 1.5	0.35 ± 0.07	203 ± 46	1.6 ± 0.3
CHOLESTYRAMINE	40.0 ± 9.0	0.37 ± 0.03	180 ± 26	1.5 ± 0.4

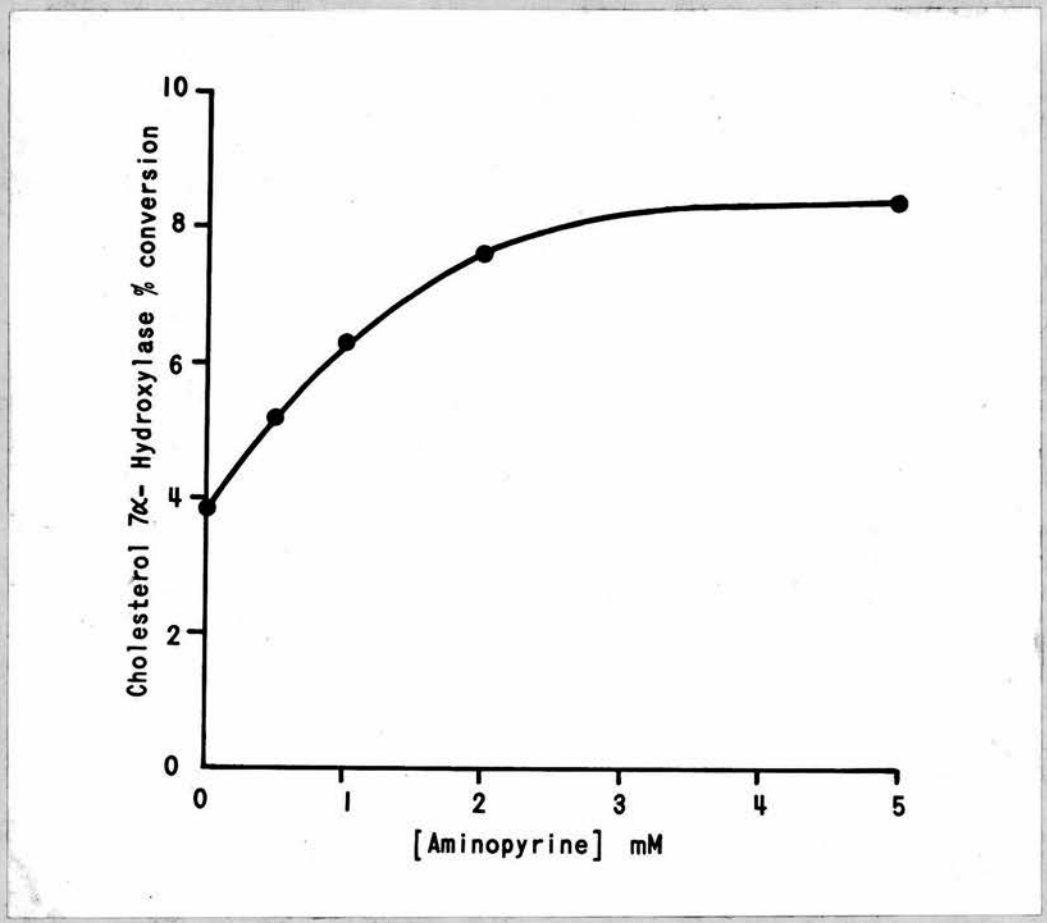


Figure 17 The effect of aminopyrine on cholesterol 7α-hydroxylase *in vitro*.

C. The Effect of Aminopyrine on Cholesterol 7α -Hydroxylase 'in Vitro'

Tephly and Mannering (1968) showed that steroids competitively inhibit the microsomal oxidation of drugs such as ethylmorphine and hexobarbital and Orrenius and Thor (1969) showed that laurate competitively inhibits the oxidation of ethylmorphine. These results suggest that those compounds which undergo oxidation by cytochrome P450 compete for the same binding site on the enzyme. Fig. 17 shows the effect on cholesterol 7α -hydroxylase of incubating microsomes in the presence of aminopyrine. The activation of cholesterol 7α -hydroxylase by aminopyrine is difficult to explain, but suggests that the drug does not bind at the same binding site on cytochrome P450 as cholesterol.

D. Phenobarbitone Treatment

The effect of administering phenobarbitone to rats for one week is shown in Table 10. Liver weight and microsomal protein content were significantly increased by the treatment in agreement with the results of Orrenius et al (1965). Although cytochrome P450 content was increased by approximately three-fold, there was no effect on the specific activity of cholesterol 7α -hydroxylase. However there was an increase in the level of the enzyme when calculated on the basis of total liver weight. Phenobarbitone treatment has a similar effect on the liver microsomal enzyme nucleoside diphosphatase (Orrenius et al 1969).

E. 3-Methylcholanthrene Treatment

3-Methylcholanthrene is a polycyclic hydrocarbon which

Table 10 The effect of phenobarbitone treatment

	Control	Phenobarbitone	Difference
Liver weight (g)	11.3 ± 2.0	15.1 ± 1.9	$P < 0.05$
Microsomal protein (mg/g liver)	11.5 ± 0.7	15.0 ± 1.7	$P < 0.001$
Cytochrome P450 (nmoles/mg protein)	0.37 ± 0.03	1.30 ± 0.13	$P < 0.001$
cholesterol 7α-hydroxylase (pmoles/min/mg protein)	13.9 ± 2.5	15.4 ± 2.0	N.S.
cholesterol 7α-hydroxylase (pmoles/min/liver)	1830 ± 241	3830 ± 516	$P < 0.01$
	n = 8	n = 8	

N.S. not significant

n = no. of animals in each group.

Table 11 The effect of 3-methylcholanthrene treatment

	Control	3-methylcholanthrene	Difference
Liver weight (g)	11.2 ± 1.5	11.1 ± 1.0	N.S.
Microsomal protein (mg/g liver)	10.7 ± 0.9	9.9 ± 1.0	N.S.
Cytochrome P450 (nmoles/mg protein)	0.47 ± 0.04	0.84 ± 0.02	$P < 0.001$
cholesterol 7α-hydroxylase (pmoles/min/mg protein	16.2 ± 4.0	15.0 ± 3.7	N.S.
cholesterol 7α-hydroxylase (pmoles/min/liver)	1923 ± 395	1804 ± 638	N.S.
	n = 4	n = 4	

N.S. not significant

n = no. of animals in each group.

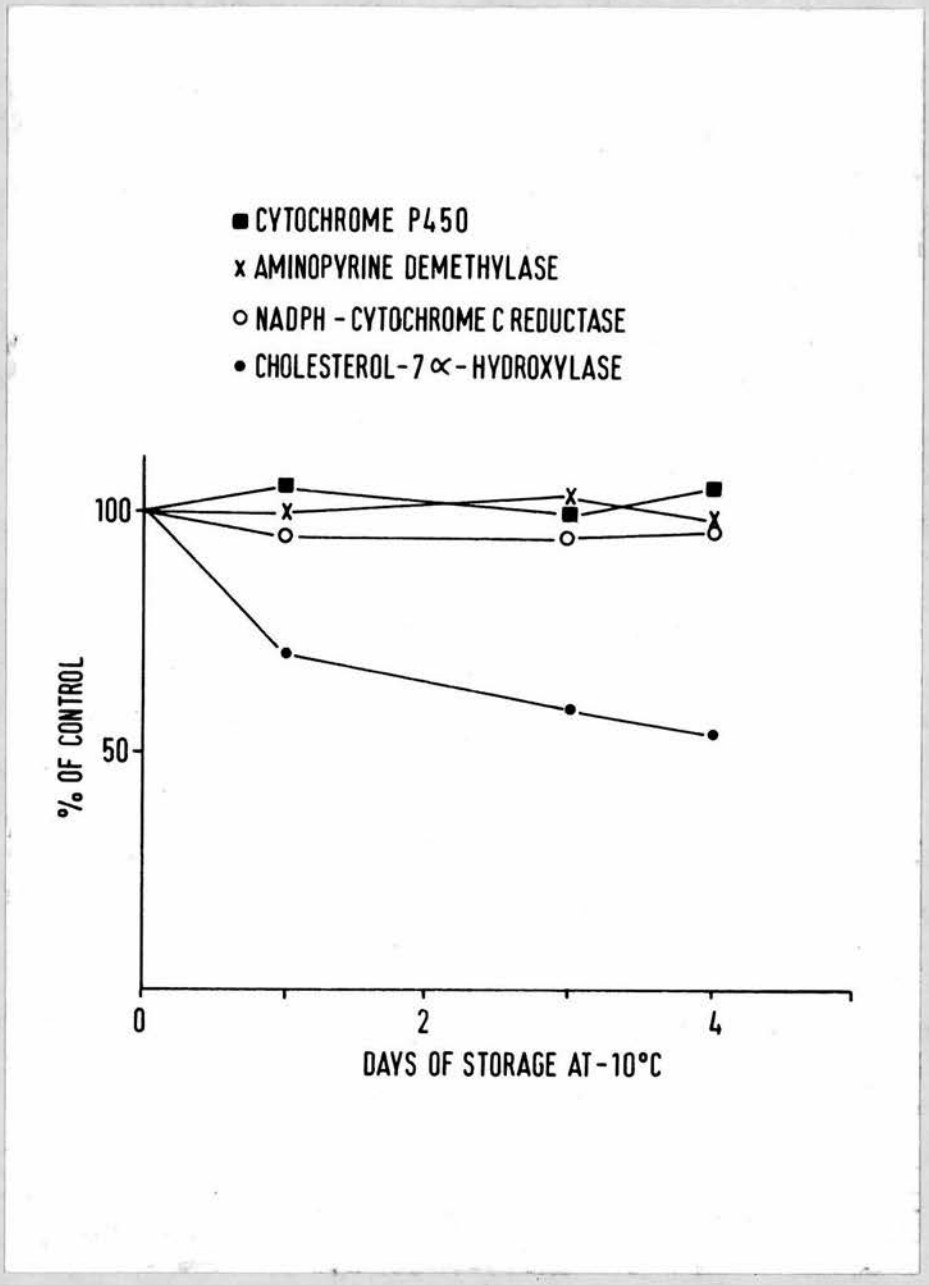


Figure 18 The effect of storing rat liver microsomes at -10°C on enzyme activity.

induces a form of rat liver cytochrome P450 which is in the high spin state (Section 1).

Table 11 shows that although this agent significantly induced microsomal cytochrome P450 levels, it had no effect on cholesterol 7α-hydroxylase activity. In contrast to phenobarbitone treatment, there was no increase in liver weight or microsomal protein content and hence the total level of enzyme per liver did not alter.

F. The Effect of Starvation

In Section 4 (Table 6) it was shown that starvation of animals for 48 hours significantly reduced the specific activity of cholesterol 7α-hydroxylase. This treatment significantly decreased liver weight and microsomal protein content, but there was no change in the cytochrome P450 content per g of liver, hence the amount of cytochrome P450 per mg of microsomal protein was increased.

The effect of different *in vivo* dietetic regimens and treatments on *in vitro* liver microsomal cholesterol 7α-hydroxylase activity and cytochrome P450 levels is summarised in Table 12, and shows the lack of correlation between these two enzymic parameters.

G. Storage of Liver Microsomes at -10°C

Conney et al (1969) showed that storage of rat liver microsomes in the deep freeze had a differential effect on the activity of testosterone hydroxylation at positions 6β, 7α and 16α. Whereas there was a progressive decrease in 6β and 16α-hydroxylation which took place over several weeks, the 7α-hydroxylase activity remained stable.

Table 12

Relationship between cytochrome P450 levels and cholesterol 7 α-hydroxylase

Treatment	Cytochrome P450 nmoles/mg protein	Cholesterol-7α - hydroxylase pmoles/min/mg protein
Control	0.35 ± 0.07	14.0 ± 1.5
Cholestyramine 4% in diet	0.37 ± 0.03	39.9 ± 9.0*
3-methyl cholanthrene 20 mg/kg i.p. for 3 days	0.84 ± 0.02*	15.0 ± 3.7
Phenobarbitone 1 mg/ml in drinking water for 6 days	1.30 ± 0.13*	15.4 ± 2.0
Starvation for 48 hrs.	0.60 ± 0.03*	5.8 ± 0.4*

*$p < 0.001$.

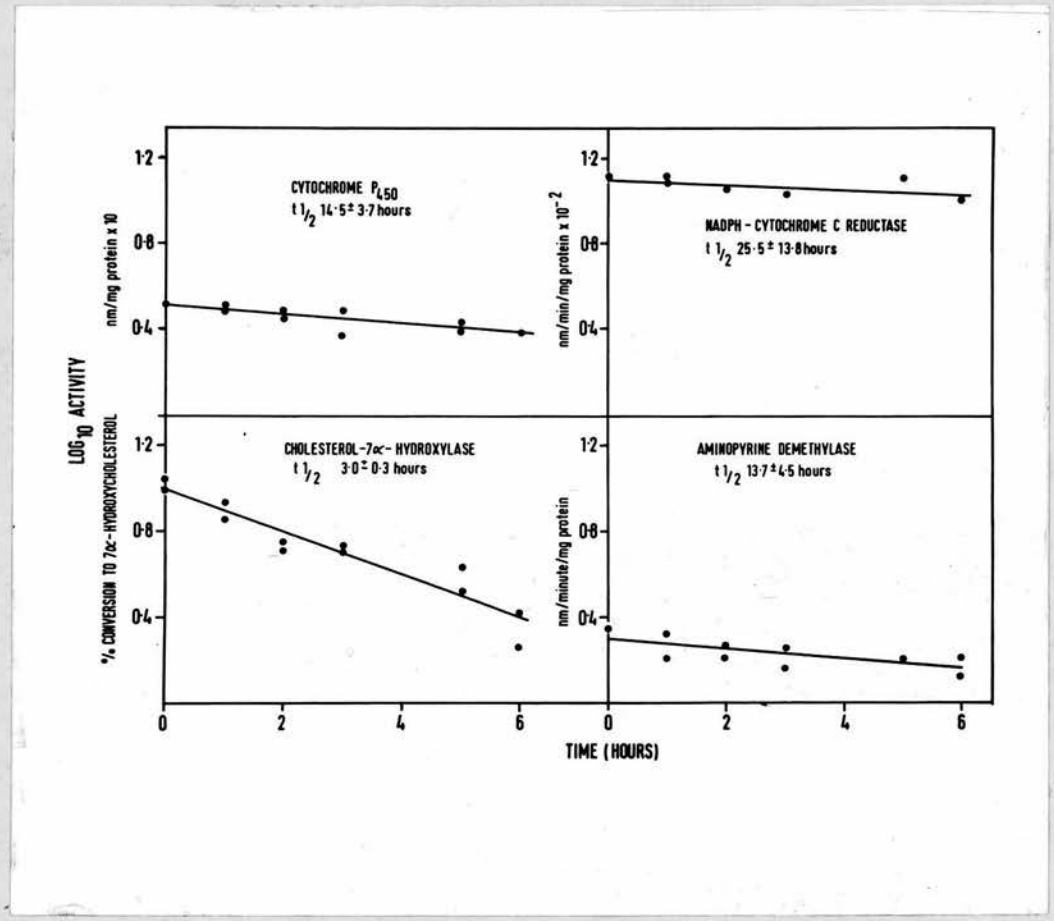


Figure 19 The effect of cycloheximide treatment on enzyme activity. The animals were given a single injection at 9.00 a.m. according to the procedure described in Section 2, part E.

The effect of storing rat liver microsomes for several days at -10°C is shown in Fig. 18. Cholesterol 7α - hydroxylase was very labile to this treatment, the activity being reduced by approximately 50% after several days, whereas aminopyrine demethylase, NADPH cytochrome c reductase and cytochrome P450 remained stable.

H. Cycloheximide Treatment

The effect of cycloheximide treatment on cholesterol 7α-hydroxylase, aminopyrine demethylase, cytochrome P450 and NADPH cytochrome c reductase is shown in Fig. 19. The dose of cycloheximide administered (5 mg/kg) is sufficient to inhibit the translation of messenger RNA into protein in rat liver by approximately 95% for 6 hours without having much effect on nuclear RNA synthesis (Harris et al 1969). Hence the time course of the enzyme activities following this treatment should give a measure of the rate of degradation of the enzyme in vivo. As shown in Fig. 19, cholesterol 7α-hydroxylase activity decayed more rapidly than the other three enzymes studied, the half-life being approximately 3 hours. This value is in agreement with that reported by Einarsson and Johansson (1968a) who found that cholesterol 7α-hydroxylase activity decayed with a half-life of 2-3 hours following puromycin treatment. Cycloheximide had no effect on rat liver microsomal cholesterol 7α-hydroxylase in vitro.

Levin and Kuntzman (1969) showed that the radioactivity incorporated into 'CO-binding particles' from (3,5-^{3}H) δ-aminolaevulinic acid (a precursor of the haem moiety) decayed biphasically, the half-lives of the two components

being 7 hours and 48 hours. They equated these two components with low spin cytochrome P450 and high spin cytochrome P450 respectively. In this experiment the measurements of cytochrome P450 following cycloheximide treatment were made over a period of only 6 hours so that a biphasic effect would not be observed.

These studies show that cholesterol 7α -hydroxylase decays more rapidly after cycloheximide treatment than does the drug hydroxylase aminopyrine demethylase, or the components of the hydroxylation system cytochrome P450 and NADPH cytochrome c reductase.

I. The Effect of Preincubating (4-^{14}C) Cholesterol with Rat Liver Microsomes on the Formation of (4-^{14}C) 7α -Hydroxycholesterol

The above experiments suggest that although cytochrome P450 may be the terminal oxidase for cholesterol 7α - hydroxylase, it does not appear to be rate-limiting. The cholesterol side chain cleavage enzyme of adrenal cortex mitochondria is also a mixed function oxidase having cytochrome P450 as the terminal electron acceptor (Simpson and Boyd 1967). The activity of this enzyme is stimulated by ACTH and the activation is not accompanied by an increase in the amount of cytochrome P450. Evidence has accumulated which suggests that this enzyme may be regulated by the amount of substrate cholesterol which is bound to the cytochrome P450 and this is in turn regulated by a cycloheximide sensitive factor which has a half-life of several minutes (Simpson *et al* 1972; Jefcoate *et al* 1972; Garren *et al* 1965). It is conceivable that a similar type

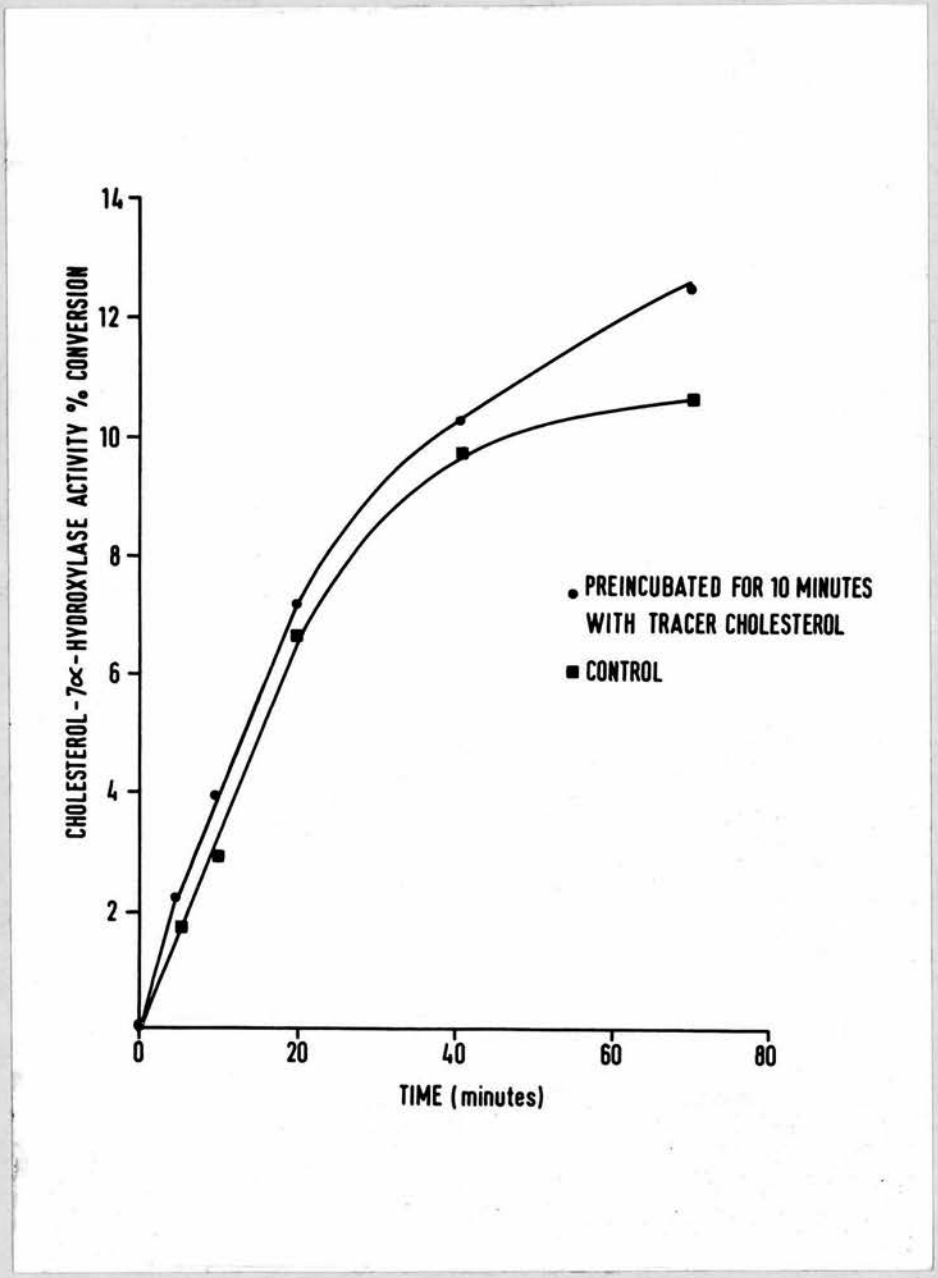


Figure 20 The effect of preincubating (4-^{14}C) cholesterol with liver microsomes on the formation of (4-^{14}C) 7α-hydroxycholesterol.

of control mechanism might exist for cholesterol hydroxylation in liver microsomes.

If the rate of cholesterol binding to the enzyme in liver microsomes is a slow step, as it appears to be in adrenal cortex mitochondria, then preincubation of radioactive cholesterol with liver microsomes prior to starting the reaction with NADPH should increase the conversion to radioactive 7α-hydroxycholesterol. This has been demonstrated for cholesterol conversion to pregnenolone in adrenal cortex mitochondria and was interpreted as indicating a slow binding of substrate to the enzyme system (Simpson et al 1972).

The effect of preincubating (4-^{14}C) cholesterol with liver microsomes on the formation of (4-^{14}C) 7α-hydroxycholesterol is shown in Fig. 20. Liver microsomes were preincubated for 10 minutes with radioactive cholesterol before adding NADPH generator. Controls were preincubated with the same volume of acetone and radioactive cholesterol was added at the same time as the NADPH generator. The percentage conversion of radioactive cholesterol to radioactive 7α-hydroxycholesterol was consistently greater in the preincubated group. To determine if this difference was statistically significant, groups of samples were preincubated for 10 minutes and the reactions were stopped 50 minutes after the addition of NADPH generator. The results are shown in Table 13 for liver microsomes obtained from a cholestyramine treated rat and from a control rat. Preincubation of the microsomes with the radioactive cholesterol caused significantly

Table 13

The effect of preincubating (4-^{14}C) cholesterol with liver microsomes on the formation of (4-^{14}C) 7α-hydroxycholesterol

	Diet	Control	Preincubated	Difference
Cholesterol 7α-hydroxylase (% conversion to 7α-hydroxycholesterol)	Soft diet	1.24 $\pm$ 0.02 (n = 5)	1.33 $\pm$ 0.03 (n = 5)	$P < 0.05$
	Cholestyramine	6.62 $\pm$ 0.13 (n = 6)	7.18 $\pm$ 0.11 (n = 6)	$P < 0.01$

n = no. of animals in each group.

greater conversion to radioactive product in each case, but the difference appeared to be greater with microsomes from the cholestyramine treated rat.

These results suggest that formation of enzyme-cholesterol complex is a slow step and that preincubation allows the radioactive substrate to equilibrate with a small pool of endogenous cholesterol which is closely bound to the enzyme. This hypothesis is not inconsistent with the results of Section 3, part F, which indicated that the radioactive cholesterol equilibrated immediately with all the endogenous substrate. The increase in percentage conversion of radioactive cholesterol which is produced by preincubation is less than 0'5%, which would correspond to less than 1 µg of endogenous cholesterol. From the results of Section 3 (part E), it is unlikely that the degree of precision of the two assays would be great enough to detect this difference. Preincubation for longer than 10 minutes did not significantly increase the formation of (4-^{14}C) 7α-hydroxycholesterol. The half-time for complete equilibration was estimated to be of the order of 5 minutes.

If formation of cholesterol-enzyme complex in liver microsomes is a slow step, as these results suggest, then this step could be rate limiting in cholesterol 7α-hydroxylation. This could be the step which is enhanced by cholestyramine treatment and which is cycloheximide sensitive and labile to freezing and thawing. This is discussed in greater detail in Section 7.

Summary to Section 5

(1) Cholestyramine feeding stimulated cholesterol 7α - hydroxylase activity without affecting cytochrome P450 concentrations, NADPH cytochrome c reductase activity or the rate of demethylation of the drug, aminopyrine.

(2) Aminopyrine activated liver microsomal cholesterol 7α -hydroxylase *in vitro*, activation being half-maximal at approximately 1 mM.

(3) Phenobarbitone treatment (1 mg/ml in the drinking water for one week) increased the concentration of liver microsomal cytochrome P450, but had no effect on the specific activity of liver microsomal cholesterol 7α - hydroxylase. However, the total activity of cholesterol 7α -hydroxylase per liver was significantly increased by this treatment.

(4) 3-Methylcholanthrene treatment (20 mg/kg body weight for three days) increased the concentration of liver microsomal cytochrome P450 but had no effect on the specific activity of cholesterol 7α -hydroxylase or on the total activity of the enzyme in the liver.

(5) Starvation for 48 hours significantly increased the content of hepatic cytochrome P450 per mg of microsomal protein but decreased the specific activity of liver microsomal cholesterol 7α -hydroxylase.

(6) Storage of liver microsomes at -10°C for several days resulted in approximately 50% reduction of liver microsomal cholesterol 7α -hydroxylase activity, but cytochrome P450, NADPH cytochrome c reductase and aminopyrine demethylase remained stable to this treatment.

(7) Cycloheximide treatment (a single injection at a dose of 5 mg/kg body weight) resulted in a rapid decay of cholesterol 7α-hydroxylase activity (half-life approximately 3 hours). Cytochrome P450, NADPH cytochrome c reductase and aminopyrine demethylase decayed at slower rates.

(8) Preincubating liver microsomes with (4-^{14}C) cholesterol for 10 minutes prior to starting the reaction with NADPH resulted in a significantly greater production of (4-^{14}C) 7α-hydroxycholesterol. This suggests that the binding of substrate cholesterol with the enzyme is a slow step.

SECTION 6

THE SPECIFICITY OF THE RAT LIVER MICROSOMAL CHOLESTEROL 7α -HYDROXYLASE

A. Introduction

The results reported in Section 5 show that there are certain important differences between cholesterol 7α -hydroxylase and aminopyrine demethylase. Although both of these enzymes require cytochrome P450 as the terminal oxidase, they appear to be regulated by different mechanisms *in vivo*. One possible explanation is that the rate of cholesterol hydroxylation is not limited by the amount of cytochrome P450 in the liver, but by another component which is specific for cholesterol. This could be a carrier protein for transporting substrate cholesterol to the oxygen activating site. Sterol carrier proteins have been implicated in hepatic cholesterol biosynthesis (Ritter and Dempsey 1971; Scallen *et al* 1971). The liver microsomal mixed function oxidase system is non-specific and from a teleological viewpoint it seems reasonable that different mechanisms should have evolved for the control of bile acid biosynthesis and the oxidation of foreign compounds. It is therefore of interest to establish the degree of specificity of the cholesterol 7α -hydroxylase enzyme.

This section concerns studies which have been made on the oxidation of certain steroid substrates and cholesterol by liver microsomes in the presence of NADPH and oxygen. The substrates chosen for study were the androgen dehydroepiandrosterone, the intermediate in steroid

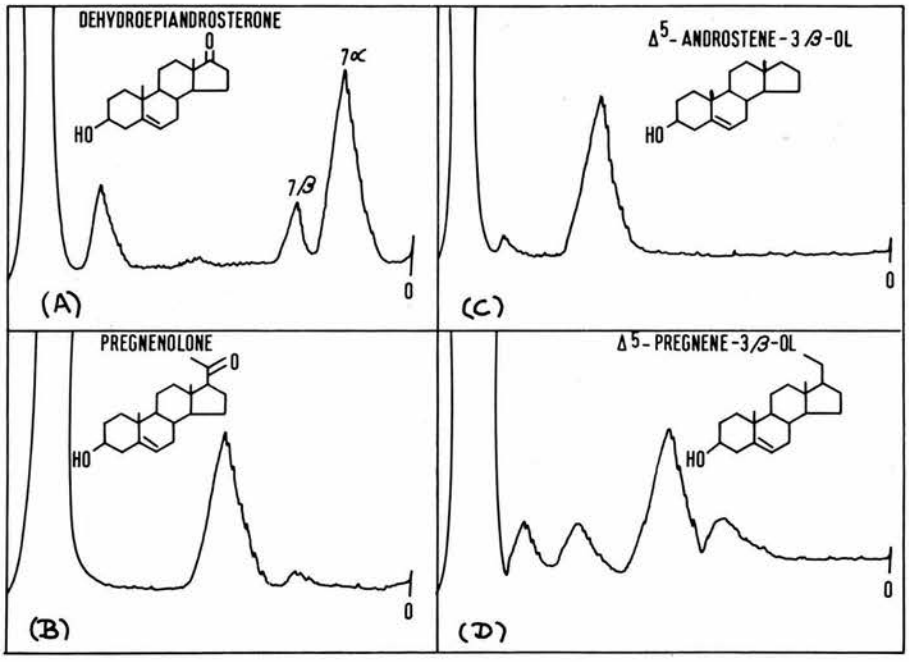


Figure 21 Radioactive scans of thin layer plates of the lipid extracts obtained after incubation of liver microsomes with (A) (4-^{14}C) dehydroepiandrosterone, (B) (4-^{14}C) pregnenolone, (C) (^{3}H) androst-5-ene-3β-ol, (D) (^{3}H) pregn-5-ene-3β-ol.

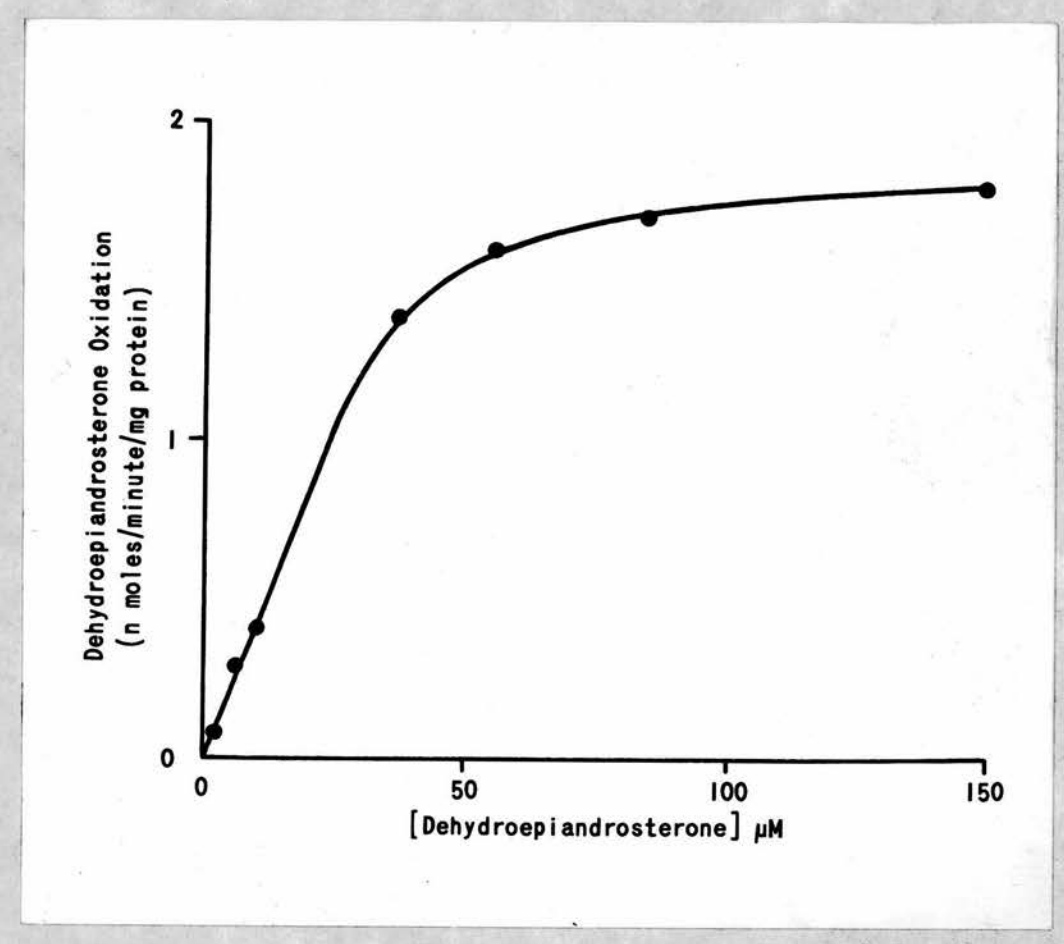


Figure 22 The effect of substrate concentration on the rate of dehydroepiandrosterone oxidation.
Microsomal protein concentration 2 mg/ml incubation time, 15 minutes.

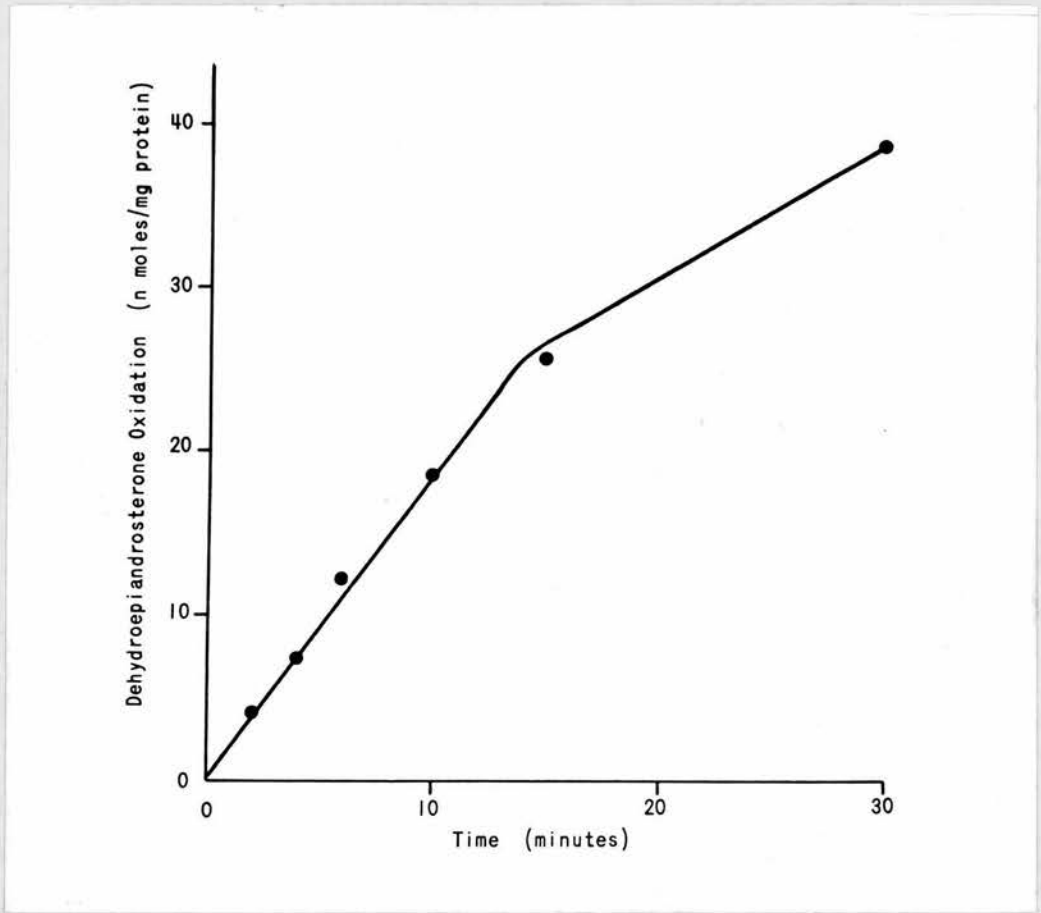


Figure 23 Time course for dehydroepiandrosterone oxidation.

hormone biosynthesis pregnenolone, and the ketone reduction products of these, androst-5-ene-3β-ol and pregn-5-ene-3β-ol respectively. These compounds have the same steroid nucleus as cholesterol (Fig. 1) but differ in the structure of the side-chain.

B. The Metabolism of Dehydroepiandrosterone, Pregnenolone, Androst-5-ene-3β-ol and Pregn-5-ene-3β-ol by Rat Liver Microsomes

The patterns of products obtained after TLC of the lipid extracts from incubations of the radioactive steroids with liver microsomes, NADPH and oxygen are shown in Fig. 21. No products were obtained when NADPH was excluded from the incubation medium or when the incubations were conducted under an atmosphere of nitrogen. No products were formed when boiled microsomes were used in the incubations. The oxidation of each of these steroids will now be considered individually.

C. The Oxidation of Dehydroepiandrosterone

The metabolism of dehydroepiandrosterone by rat liver microsomes has been studied by Sulcova and Starka (1968) who reported the formation of the 7 -hydroxylated epimers, and by Heinrichs and Colas (1968) who reported in addition the formation of the 16α-hydroxylated product. These workers employed colorimetric methods to measure product formation.

The pattern of products obtained following incubation of (4-^{14}C) dehydroepiandrosterone with rat liver microsomes is shown in Fig. 21A. Three products were detected.

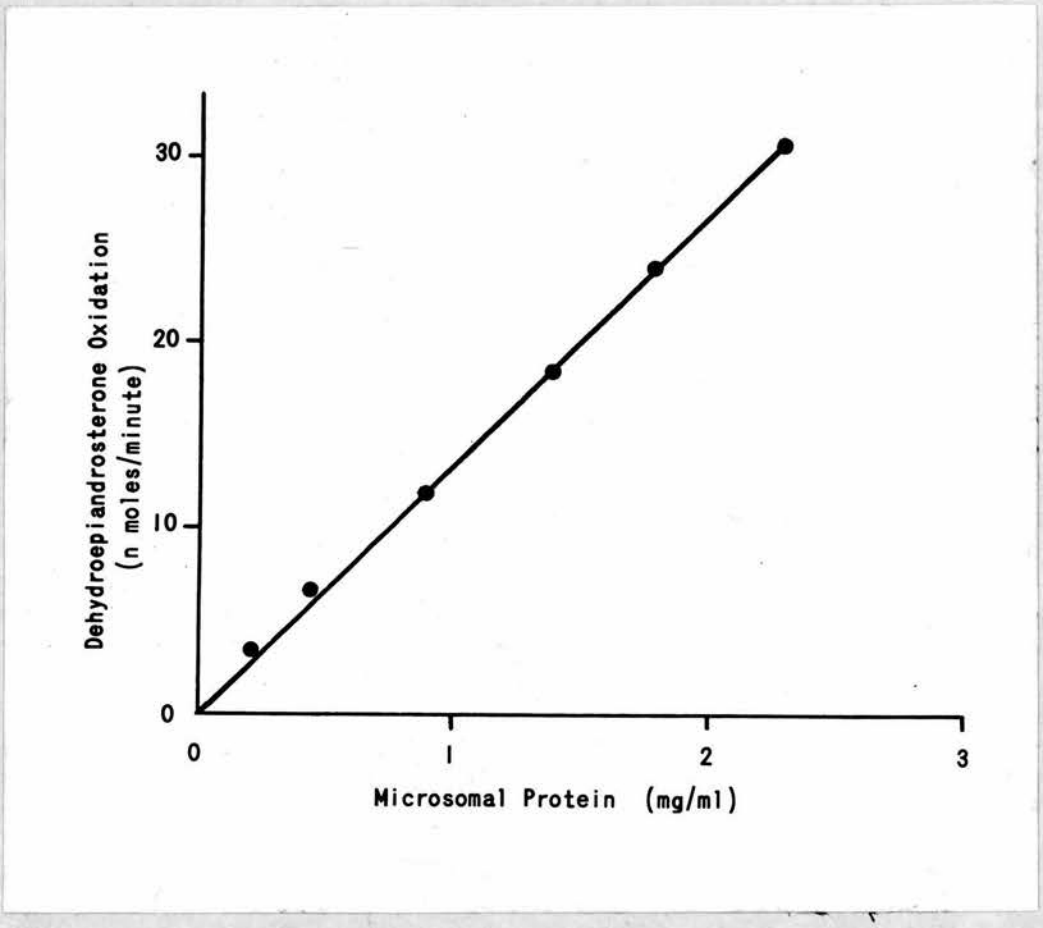


Figure 24 The effect of microsomal protein concentration on dehydroepiandrosterone oxidation.

These have been tentatively identified as the 16α , 7α and 7β-hydroxylated steroids (in decreasing order of Rf value) by comparing their behaviour on TLC in different solvent systems with the standard reference compounds. The major metabolite was the 7α-hydroxylated steroid, and the characteristics of its enzymic formation have been investigated.

Fig. 22 shows that the enzyme was half-saturated when the substrate concentration was approximately 25 μM. The reaction was first order with respect to microsomal protein concentration (Fig. 24) and was linear for approximately 15 minutes at 37°C (Fig. 23).

The inclusion of 10 mM β-mercaptoethylamine in the incubation medium did not affect the formation of any of the products of dehydroepiandrosterone metabolism. As β-mercaptoethylamine inhibits the formation of 'autoxidation products' of cholesterol and lipid peroxides by liver microsomes (Grimwade et al 1971), this suggests that the products of dehydroepiandrosterone metabolism are not formed by a peroxidative mechanism. Carbon monoxide was found to markedly inhibit the formation of all three products, implicating the involvement of cytochrome P450 in the hydroxylation mechanism.

D. The Oxidation of Pregnenolone

Starka et al (1966) reported that pregnenolone was hydroxylated in the 7α-position by rat liver microsomes in the presence of NADPH and oxygen. The pattern of products obtained when (4-^{14}C) pregnenolone was incubated with liver microsomes and cofactors is shown in Fig. 21B.

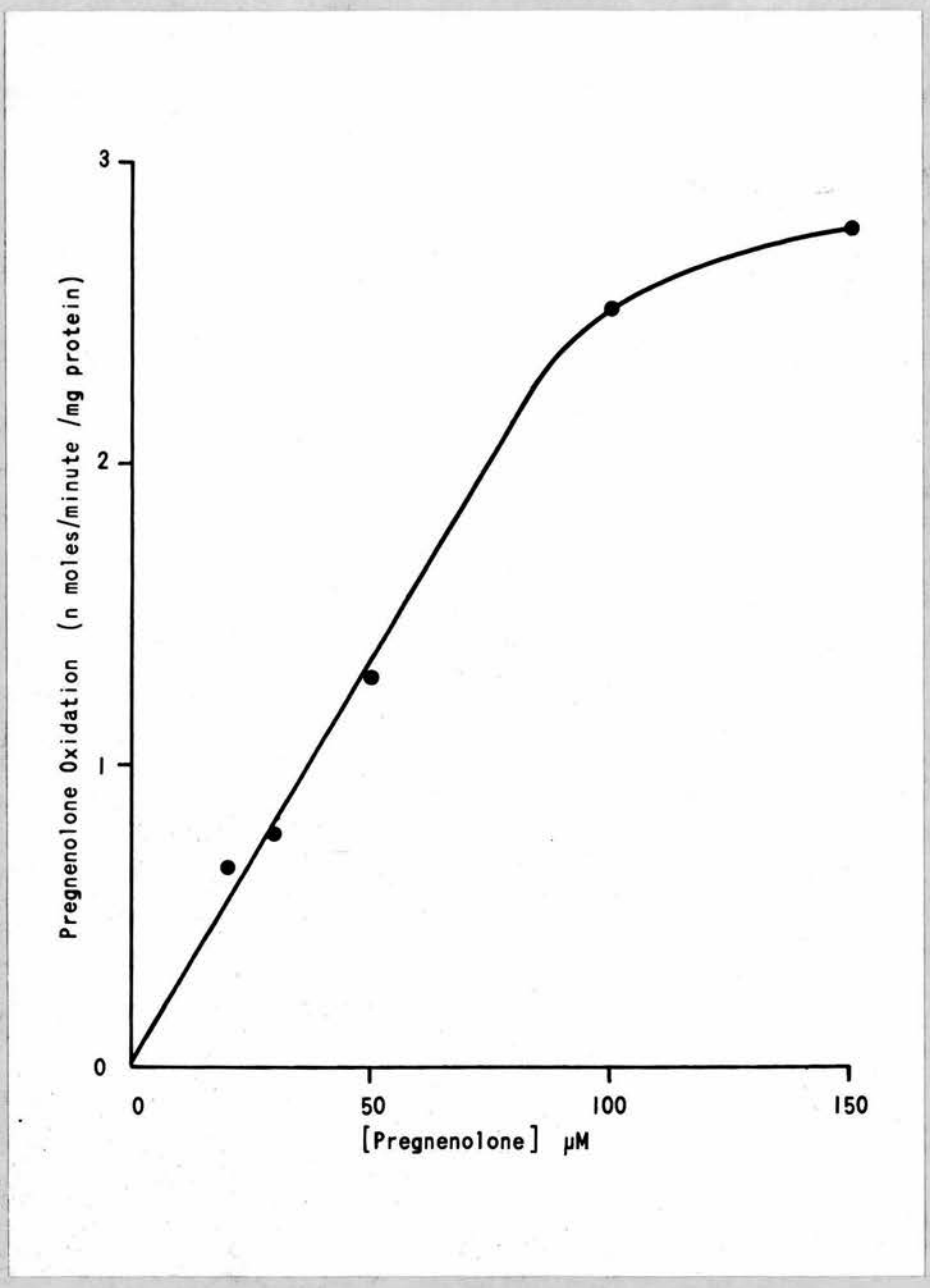


Figure 25 The effect of substrate concentration on the rate of pregnenolone oxidation.
Microsomal protein concentration, 2 mg/ml. Incubation time 15 minutes

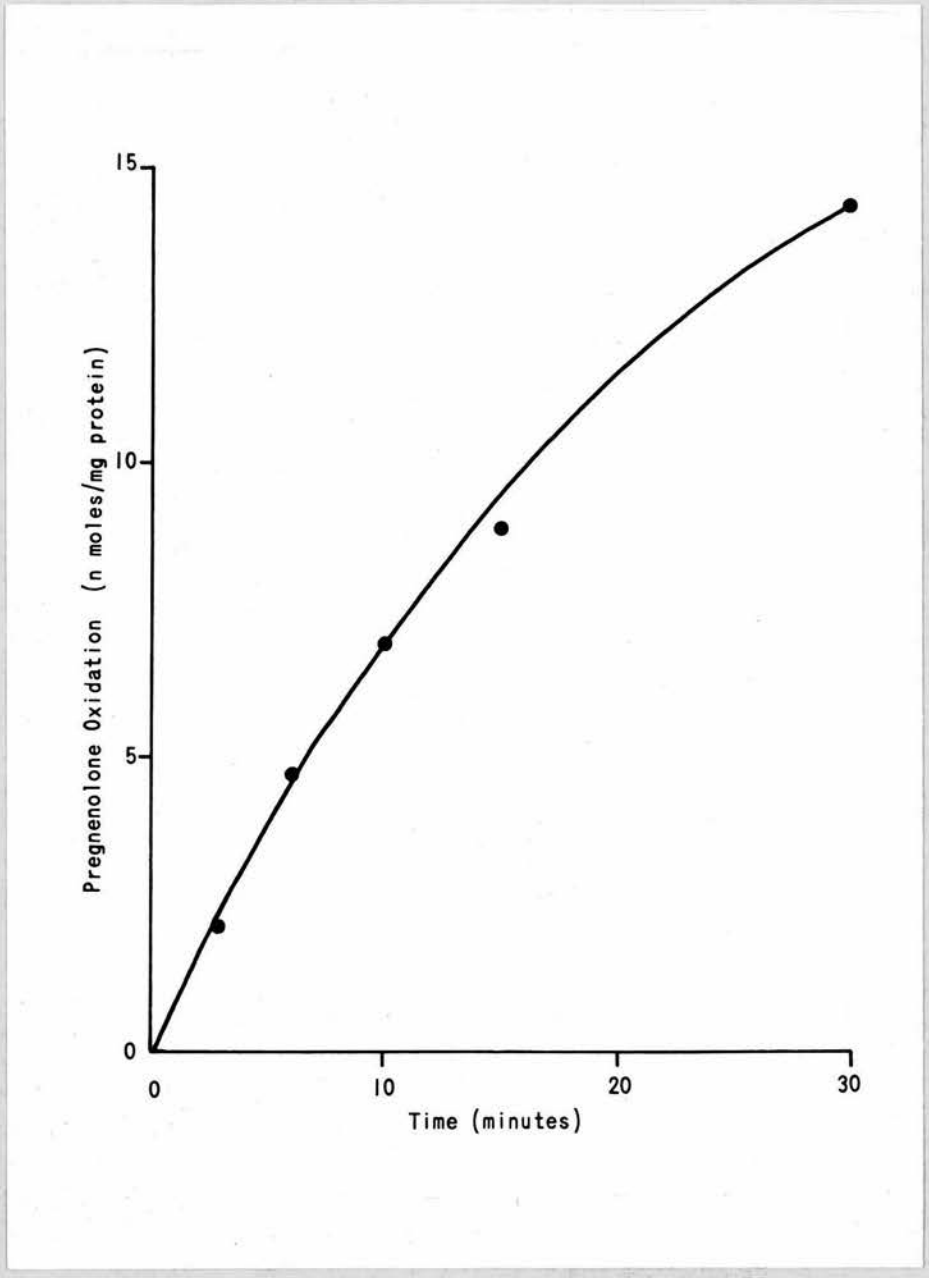


Figure 26 . Time course for pregnenolone oxidation.

Microsomal protein concentration 2 mg/ml.

Substrate concentration, 150 μM

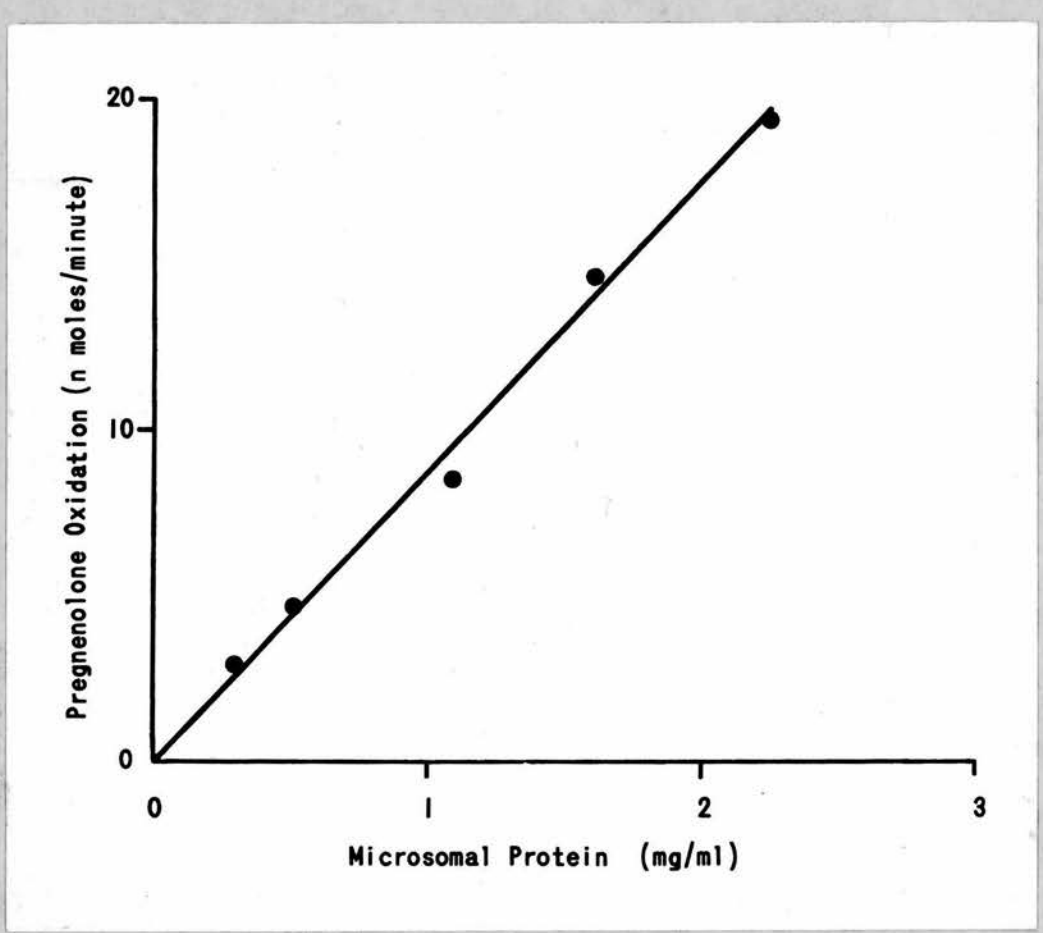


Figure 27 The effect of microsomal protein concentration on pregnenolone oxidation.

Substrate concentration, 150 μM, incubation time, 15 minutes.

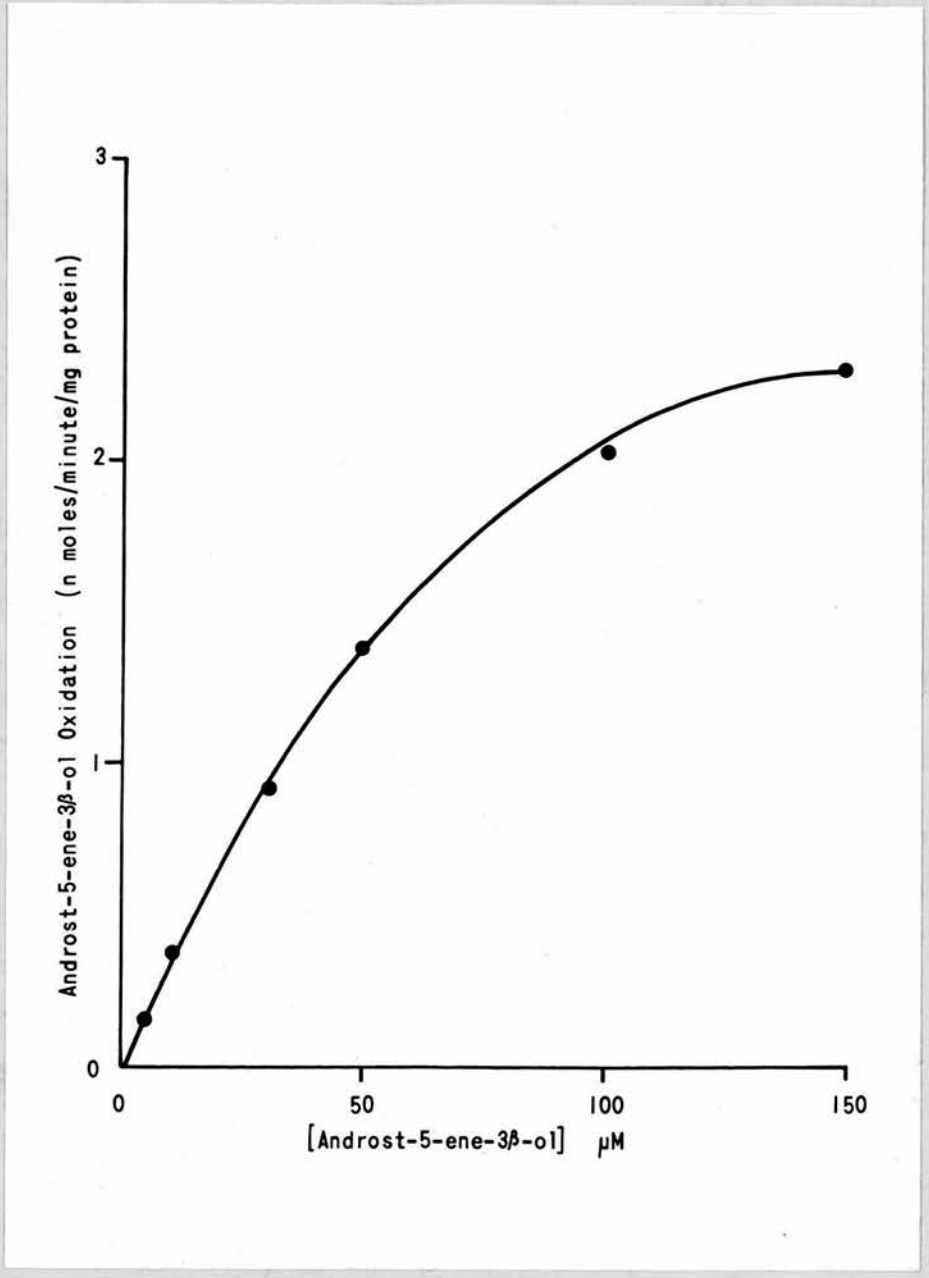


Figure 28 The effect of substrate concentration on the rate of androst-5-ene-3β-ol oxidation.

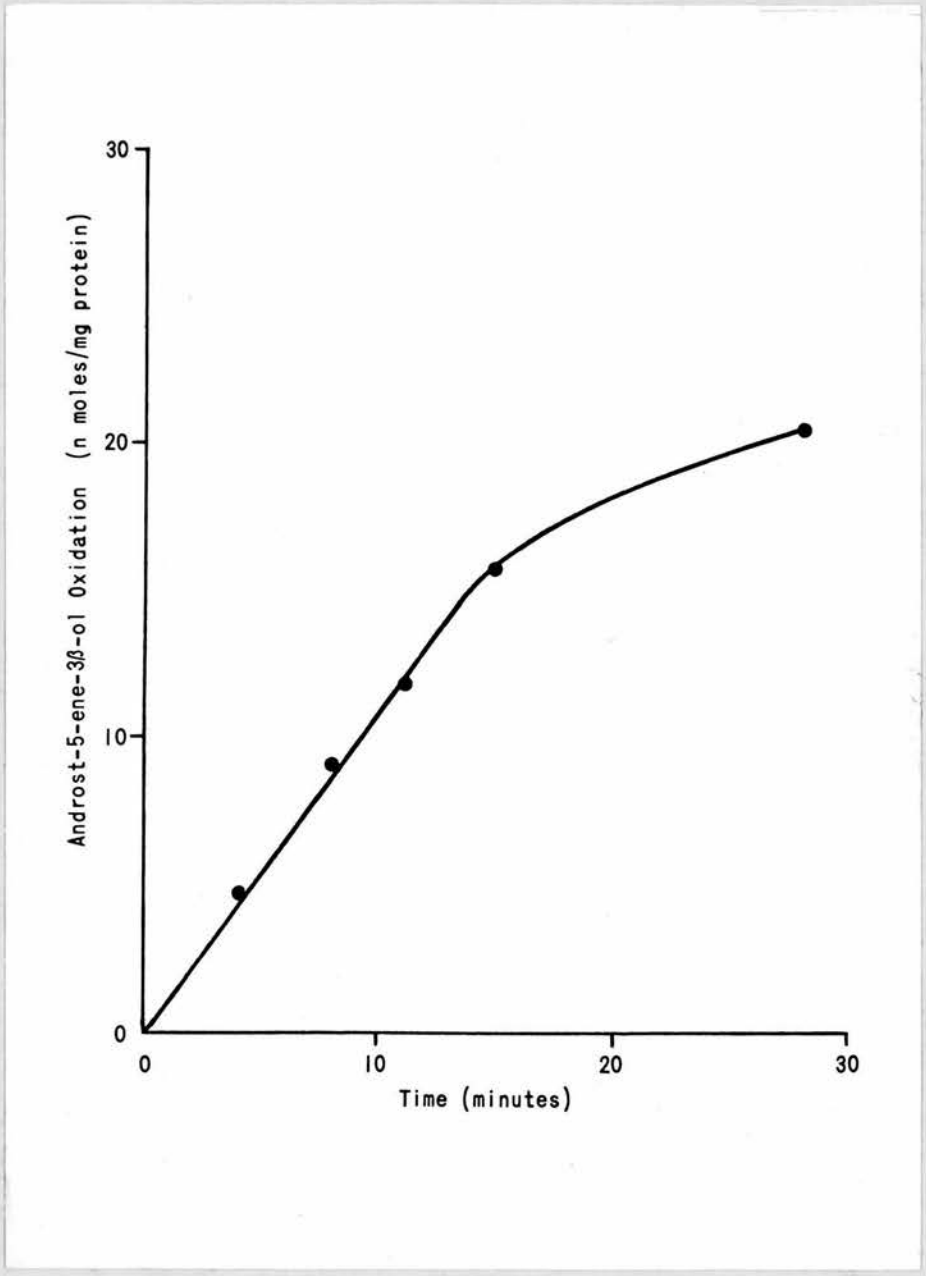


Figure 29 Time course for androst-5-ene-3β-ol oxidation.

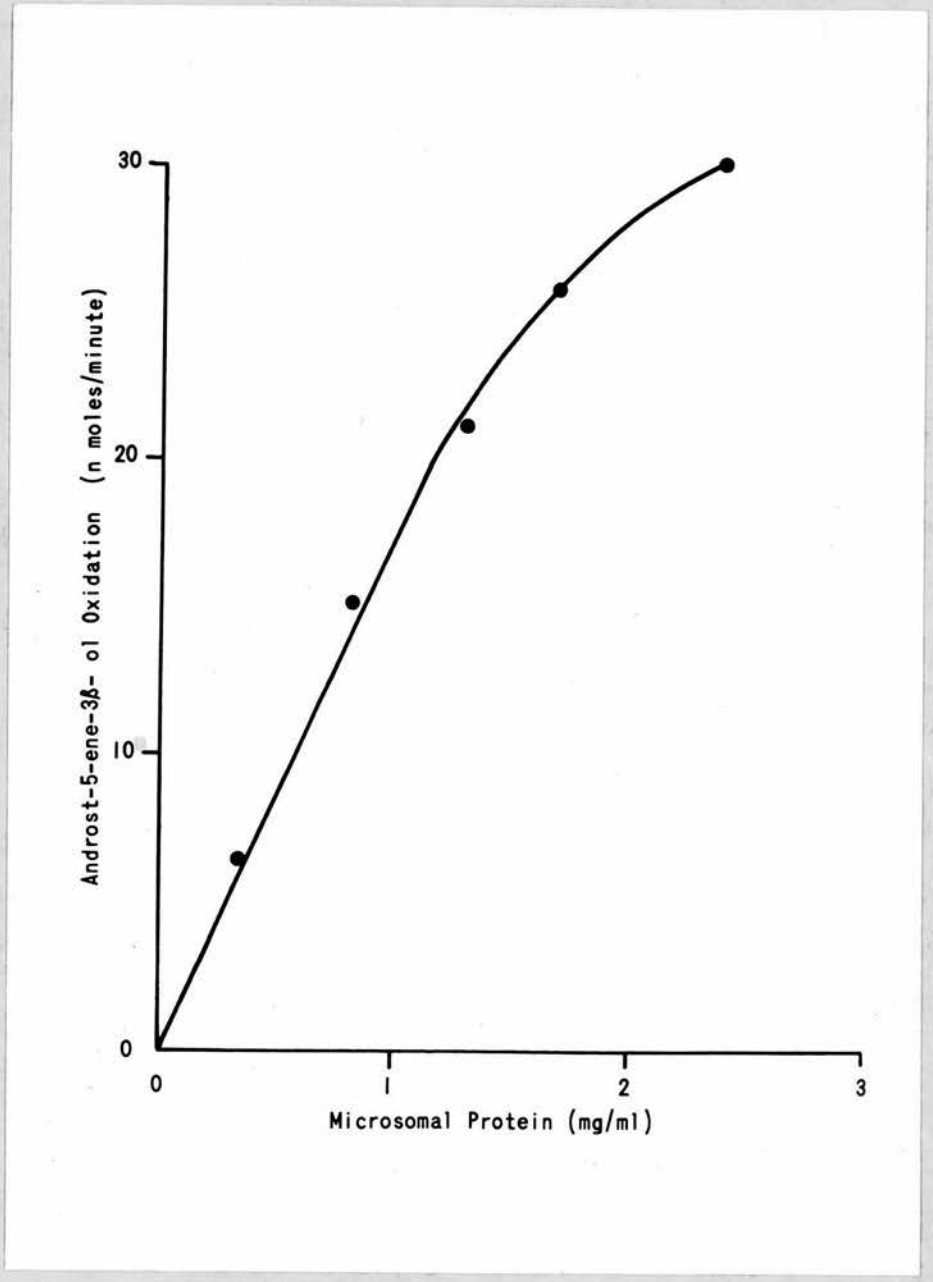


Figure 30 The effect of microsomal protein concentration on androst-5-ene-3β-ol oxidation.

When the major product was rechromatographed in different solvent systems it failed to resolve into more than one peak. When (7α -^{3}H) pregnenolone was used instead of (4-^{14}C) pregnenolone there was no loss of tritium, suggesting that this product is not the 7α-hydroxylated derivative, however it has not been identified. The rate of formation of this product was half-maximal at a substrate concentration of approximately 50 μM as shown in Fig. 25. The reaction rate was linear for approximately 15 minutes at 37°C (Fig. 26) and was first order with respect to microsomal protein concentration (Fig. 27). Product formation was not affected by β-mercaptoethylamine suggesting that a peroxidative mechanism was not involved.

E. The Oxidation of Androst-5-ene-3β-ol

Further chromatography of the product obtained from the liver microsomal metabolism of this steroid (Fig. 21C) failed to resolve it into more than one peak, however this compound has not been identified. The enzyme was half-saturated at a substrate concentration of approximately 40 μM (Fig. 28). The time course for the reaction at 37°C is shown in Fig. 29 and the effect of microsomal protein concentration in Fig. 30. As with the previous two steroid substrates discussed, the product formation was not affected by β-mercaptoethylamine.

F. The Oxidation of Pregn-5-ene-3β-ol

This steroid was metabolised to at least four products when incubated with liver microsomes, NADPH and oxygen (Fig. 21D). None of these have been positively identified.

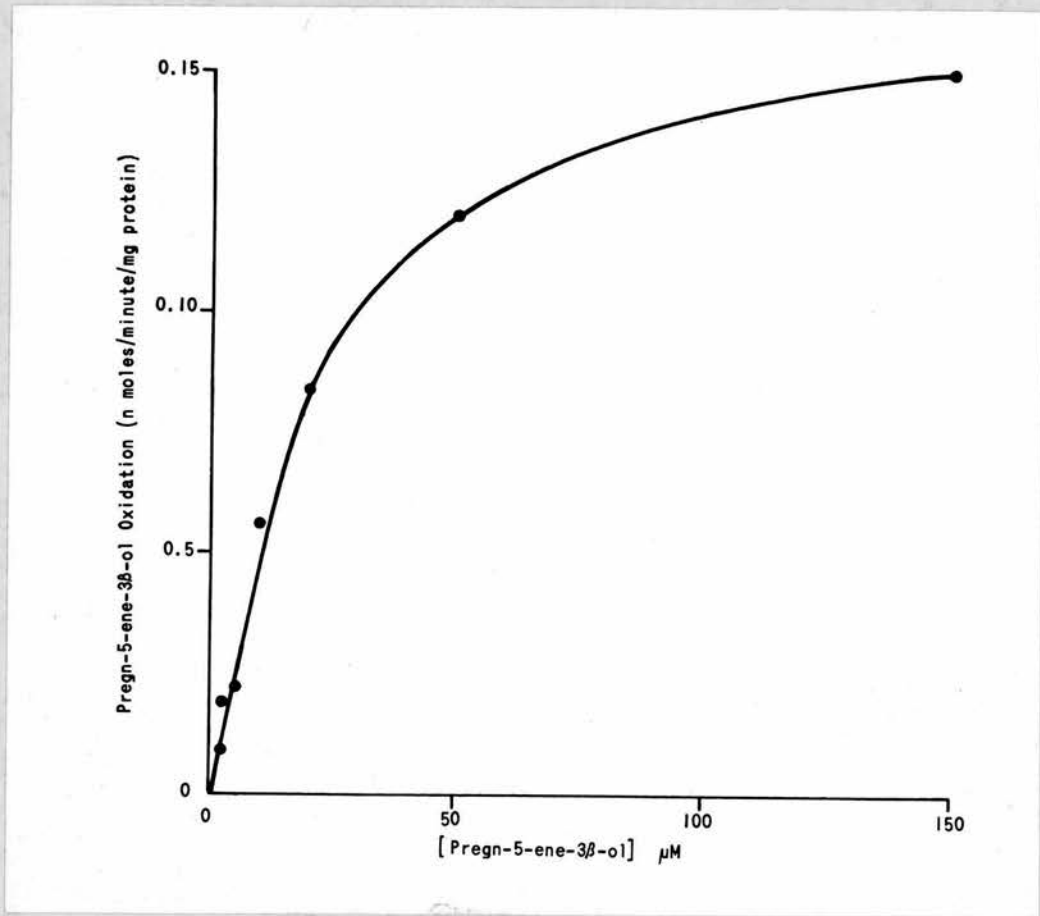


Figure 31 The effect of substrate concentration on the rate of pregn-5-ene-3β-ol oxidation.

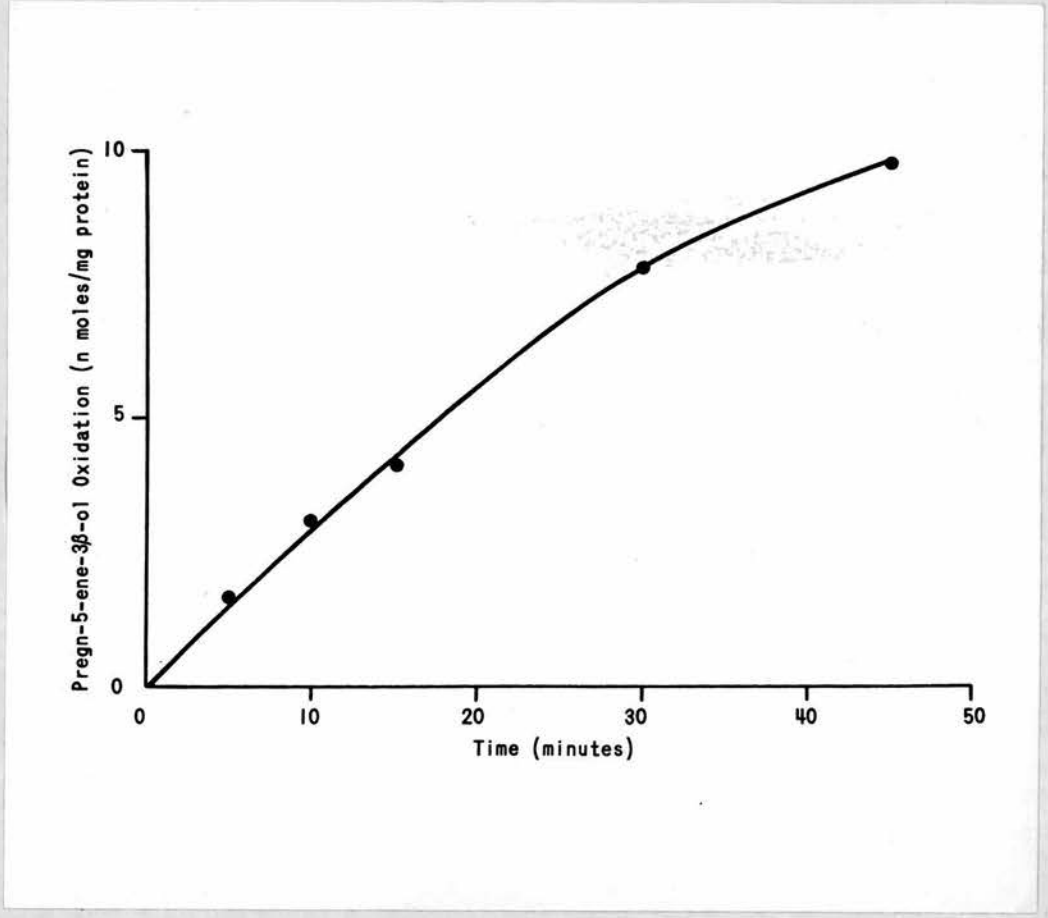


Figure 32 Time course for pregn-5-ene-3β-ol oxidation. Substrate concentration 75 μM.

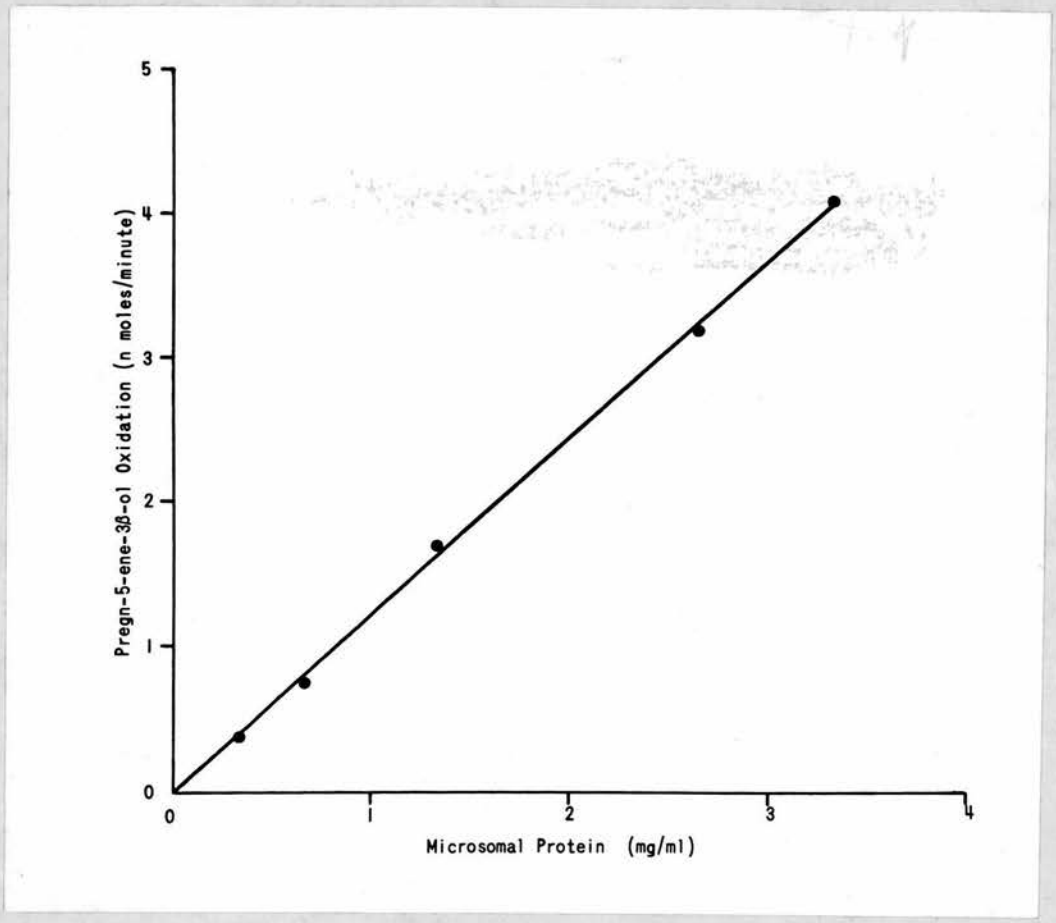


Figure 33 The effect of microsomal protein concentration on pregn-5-ene-3β-ol oxidation.

Substrate concentration 75 μM.
Incubation time 15 minutes.

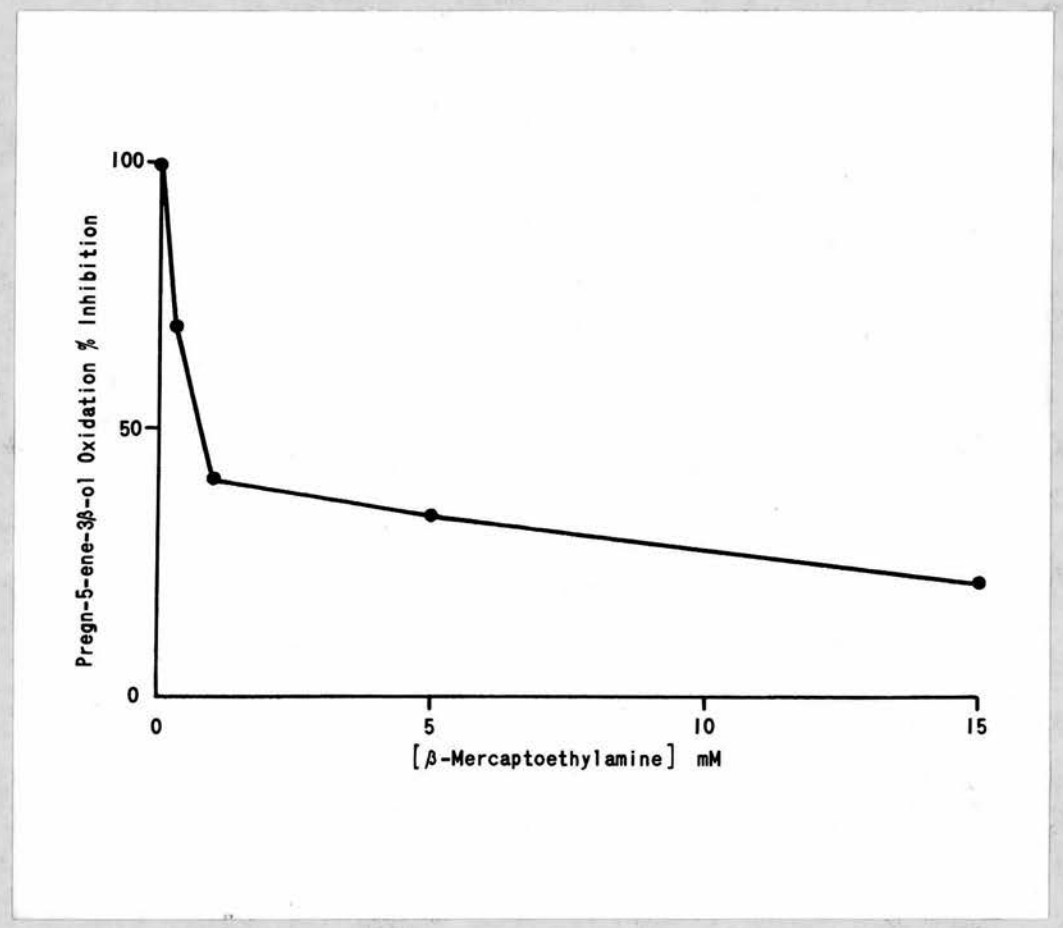


Figure 34 The effect of β-mercaptoethylamine on pregn-5-ene-3β-ol oxidation *in vitro*.

The formation of the major product (Rf = 0'48, Fig. 21D) was investigated. The reaction was half-saturated at a substrate concentration of approximately 15 μM (Fig. 31). The time course for the reaction is shown in Fig. 32 and the effect of increasing microsomal protein concentration in Fig. 33. The formation of all products was found to be markedly inhibited by incorporating β-mercaptoethylamine into the incubation medium. Fig. 34 shows the effect of increasing the concentration of this thiol on the formation of the major product. This suggests that pregn-5-ene-3β-ol might be oxidised by a peroxidative mechanism, similar to the formation of 7-ketocholesterol from cholesterol which is also inhibited at the same concentrations of β-mercaptoethylamine (Mitton et al 1971).

G. The Binding of Dehydroepiandrosterone, Pregnenolone, Androst-5-ene-3β-ol and Pregn-5-ene-3β-ol to Rat Liver Microsomes

Substrates for the cytochrome P450-dependent mixed function oxidase system of liver microsomes produce a characteristic difference spectrum when added to oxidised suspensions of liver microsomes (Section 1). The steroid substrates whose oxidative metabolism has been studied were tested for the production of difference spectra with rat liver microsomes.

Type I difference spectra were produced by dehydroepiandrosterone, pregnenolone, and androst-5-ene-3β-ol, but pregn-5-ene-3β-ol gave no difference spectrum. The binding constants (Ks) for these steroids are given in Table 14.

Table 14

The binding of sterols to liver microsomal cytochrome P450

Sterol	Ks(μM)
Dehydroepiandrosterone	0.96 ± 0.10
Pregnenolone	1.16 ± 0.10
Androst-5-ene-3β-ol	0.73 ± 0.05
Pregn-5-ene-3β-ol	-

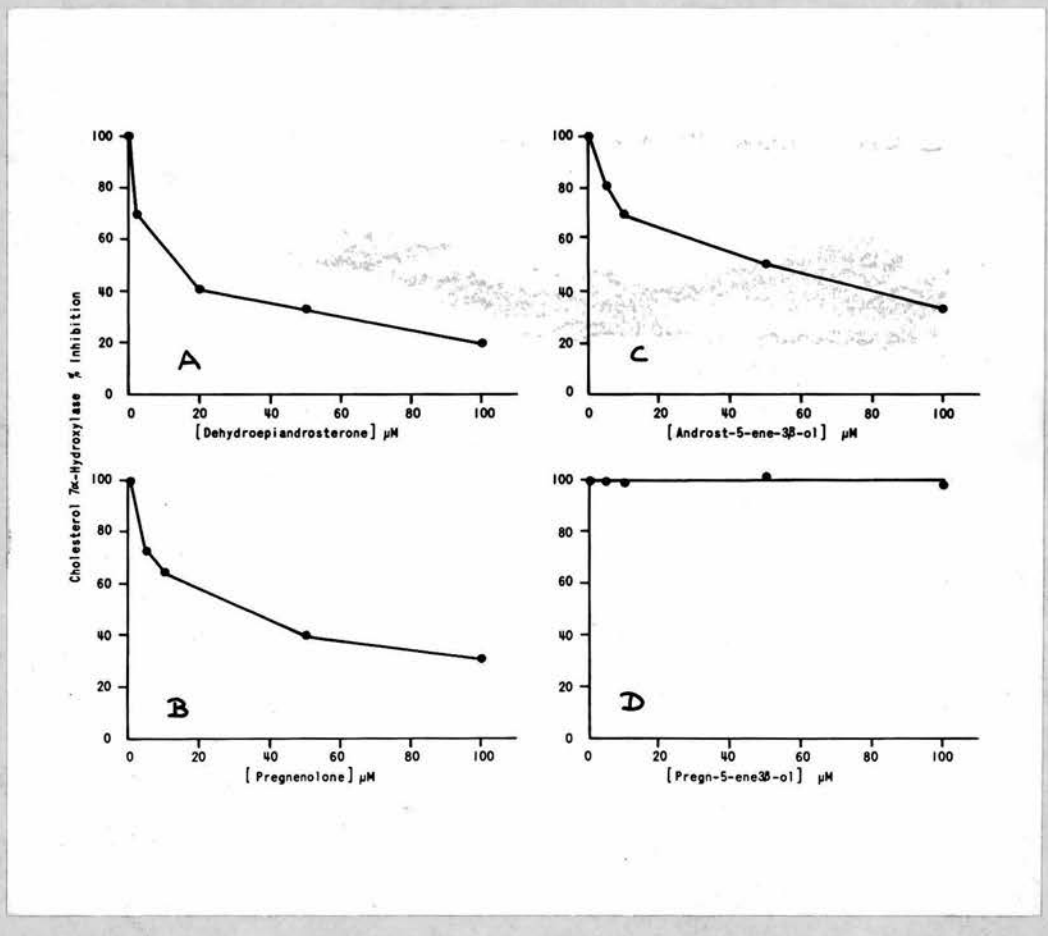


Figure 35 The effect of (A) dehydroepiandrosterone, (B) pregnenolone, (C) androst-5-ene-3 β-ol and (D) pregn-5-ene-3 β-ol on cholesterol 7α -hydroxylase *in vitro*.

This suggests that the oxidation of the former three steroids might involve cytochrome P450 and that the oxidation of pregn-5-ene-3β-ol may be peroxidative as it is inhibited by β-mercaptoethylamine.

H. The Effect of Dehydroepiandrosterone, Pregnenolone, Androst-5-ene-3β-ol and Pregn-5-ene-3β-ol on Cholesterol 7α-Hydroxylase 'In Vitro'

Fig. 35 shows the effect of these steroids on rat liver microsomal cholesterol 7α-hydroxylase in vitro. Dehydroepiandrosterone, pregnenolone and androst-5-ene-3β-ol all inhibited, whereas pregn-5-ene-3β-ol had no effect at any of the concentrations tested.

Table 15 summarises some of the properties of these steroids with respect to rat liver microsomes. Pregn-5-ene-3β-ol differs from the other three steroids in that its oxidation is inhibited by β-mercaptoethylamine and that it does not inhibit cholesterol 7α-hydroxylase or form a Type I difference spectrum with liver microsomes.

I. The Effect of Cholestyramine Feeding on Steroid Metabolism

The effect of three of these steroids in inhibiting cholesterol 7α-hydroxylase suggests that they might be metabolised by the same enzyme system. If this is so, then cholestyramine feeding, which induces cholesterol 7α-hydroxylase activity, should also stimulate the oxidation of these steroids.

Table 16 shows the effect of cholestyramine treatment on the oxidation of cholesterol, dehydroepiandrosterone, pregnenolone, androst-5-ene-3β-ol and pregn-5-ene-3β-ol.

Table 15

Sterol	Inhibits cholesterol-7α-hydroxylase	Oxidation inhibited by 10 mM β-mercaptoethylamine	Forms Type I difference spectrum with liver microsomes
Dehydroepiandrosterone	+	-	+
Pregnenolone	+	-	+
Androst-5-ene-3β-ol	+	-	+
Pregn-5-ene-3β-ol	-	+	-

Table 16 The effect of cholestyramine treatment on the rate of sterol oxidation by rat liver microsomes

Sterol	Rate of oxidation nmoles/min/mg protein		No. of animals	Difference
	Control	Cholestyramine treated		
Cholesterol	0.0140 ± 0.0015	0.0399 ± 0.0090	8	$P < 0.001$
Dehydroepiandrosterone	1.05 ± 0.35	1.14 ± 0.10	8	N.S.
Pregnenolone	1.06 ± 0.19	0.92 ± 0.07	8	N.S.
Androst-5-ene-3β-ol	0.93 ± 0.17	0.95 ± 0.10	8	N.S.
Pregn-5-ene-3β-ol	0.15 ± 0.03	0.20 ± 0.04	8	N.S.

N.S. Not significant

Dehydroepiandrosterone, pregnenolone and androst-5-ene-3β-ol (150 μM) and pregn-5-ene-3β-ol (75 μM) were incubated for 15 minutes at 37°C, as described in Section 2 (Part R).

Cholesterol 7α-hydroxylase was significantly stimulated, however there was no effect on the rate of oxidation of any of the other steroids tested. This suggests that these steroids are metabolised by a different enzyme system from cholesterol, or that there is a different rate determining step. It is interesting to note that the rate of metabolism of dehydroepiandrosterone, pregnenolone and androst-5-ene-3β-ol is approximately two orders of magnitude greater than cholesterol 7α-hydroxylase activity.

Summary to Section 6

(1) The metabolism by liver microsomes of four steroids having the same ring structure as cholesterol (3β-hydroxy, Δ^5 unsaturated) but differing in the structure of the side-chain has been investigated. These are dehydroepiandrosterone, pregnenolone, androst-5-ene-3β-ol and pregn-5-ene-3β-ol (Fig. 1).

(2) Dehydroepiandrosterone was metabolised to three products, tentatively identified as the 7α, 7β and 16α hydroxylated derivatives. The rate of formation of the major product, 3β, 7α-dihydroxyandrost-5-ene-17-one, was investigated. The formation of all of these products was inhibited by carbon monoxide.

(3) Pregnenolone was metabolised to at least one major product which has not been identified.

(4) Androst-5-ene-3β-ol was metabolised to at least one product which has not been identified.

(5) Pregn-5-ene-3β-ol was metabolised to at least four unidentified products. The rate of formation of the major product was investigated.

(6) The formation of all of these products required NADPH and oxygen. The oxidation of dehydroepiandrosterone, pregnenolone and androst-5-ene-3β-ol was not affected by β-mercaptoethylamine, but pregn-5-ene-3β-ol oxidation was markedly inhibited by this thiol.

(7) Dehydroepiandrosterone, pregnenolone and androst-5-ene-3β-ol formed Type I difference spectra with suspensions of liver microsomes, but pregn-5-ene-3β-ol did not form a difference spectrum.

(8) Dehydroepiandrosterone, pregnenolone and androst-5-ene-3β-ol all inhibited cholesterol 7α-hydroxylase activity <u>in vitro</u> but pregn-5-ene-3β-ol had no effect.

(9) Rats fed cholestyramine experienced a marked induction of liver microsomal cholesterol 7α-hydroxylase whereas there was no effect on the rate of oxidation of the other steroids tested.

SECTION 7

DISCUSSION

The steady state concentration of total body cholesterol in mammals is governed by homeostatic mechanisms which vary from species to species (review by Dietschy and Wilson 1970). Most tissues have the capacity to synthesise cholesterol but in the rat the liver accounts for approximately 80% of the total body production (Dietschy and Siperstein 1967). Cholesterol is excreted from the body mainly in the form of bile acids and faecal neutral sterols, each pathway accounting for approximately 50% of the total under normal conditions. On a sterol free diet, the rates of cholesterol synthesis and catabolism are approximately equal, but when cholesterol is supplied in the diet, hepatic cholesterologenesis is inhibited. This regulatory mechanism appears to operate in all species, control taking place at the level of the enzyme HMG CoA reductase (Siperstein and Fagan 1966). When the dietary intake is such that inhibition of endogenous synthesis is not sufficient to prevent total body cholesterol from increasing, other regulatory mechanisms operate. In man, absorption from the gastrointestinal tract is limited and suppression of hepatic HMG CoA reductase activity is probably sufficient to prevent a significant increase in whole body cholesterol content. In the rat and in the dog, there does not appear to be a limit on intestinal absorption, and excess dietary cholesterol is compensated by increasing bile acid output. Fig. 15 demonstrates that when rats were maintained on a diet containing 1% cholesterol, the total liver cholesterol

content increased by three-fold after four to five days, until a new steady-state level was attained. This excess is thought to occur mainly in the form of esterified cholesterol, the free cholesterol level remaining constant (Gould, 1955). The rabbit appears to lack the ability to accelerate bile acid production in response to increased cholesterol absorption, hence when this species is maintained on a diet high in cholesterol, the total amount of this sterol in the body increases indefinitely. Hence in the rat and in the dog, bile acid synthesis is an important regulatory mechanism in maintaining total body cholesterol levels constant.

The demonstration by Eriksson in 1957 that the drainage of bile from a bile fistula rat caused an increase in the rate of production of bile acids, led to the hypothesis that bile acids control their own rate of synthesis. In 1967, Danielsson *et al* showed that biliary diversion was accompanied by an increase in the activity of the cholesterol 7α-hydroxylase enzyme. This discovery, plus the fact that no intermediate in the pathway of cholesterol to bile acids accumulate to any extent in the liver, suggested that 7α-hydroxycholesterol formation was the rate-determining step in bile acid biosynthesis. This is supported by the finding of Shefer *et al* (1970) that when taurodeoxycholate was infused intraduodenally into rats with a biliary fistula, the conversion of radioactive cholesterol into bile acids was markedly inhibited, whereas there was no effect on the rate of conversion of radioactive 7α-hydroxycholesterol into bile acids. Hence the mechanisms which

regulate the activity of the enzyme, cholesterol 7α -hydroxylase, *in vivo* will also control the rate at which cholesterol is converted into bile acids. The object of this research has been to study the manner in which this enzyme is controlled *in vivo*, with a view to understanding the mechanisms which regulate cholesterol catabolism in rat liver.

As discussed in sections 1 and 3, the radioactive tracer assay used by workers in this and other laboratories to measure the activity of cholesterol 7α -hydroxylase is fraught with assumptions regarding substrate pool sizes and equilibration of tracer cholesterol. An unambiguous method was established which measured directly the total mass of product, 7α -hydroxycholesterol, formed in the reaction from endogenous cholesterol using a gas liquid chromatographic technique. Fig. 12 shows that the observed rate of formation of 7α -hydroxycholesterol in liver microsomal incubations was approximately the same whether measured by the radioactive assay or by the GLC assay. This suggests that the tracer cholesterol added in acetone solution equilibrates rapidly with the endogenous cholesterol of liver microsomes, the rate of equilibration being greater than the rate of the hydroxylation reaction. The effect of adding significant amounts of exogenous cholesterol was not investigated, nor was the effect of adding the cholesterol in a different medium. Mosbach and his coworkers (Shefer *et al* 1968) added significant amounts of endogenous cholesterol to their incubations and Mitropoulos and Balasubramaniam (1972) added radioactive cholesterol suspended in Tween 80. These latter

workers showed that in their experiments (4-^{14}C) cholesterol added in Tween 80 did not equilibrate uniformly with all the endogenous cholesterol. This discrepancy might be due to the mode of addition of radioactive substrate, or to a difference in the method used to prepare liver microsomes. In our experiments, however, it appears that measurement of the initial rate of conversion of radioactive tracer cholesterol into radioactive 7α -hydroxycholesterol, coupled with measurement of the total substrate pool, is a valid means of estimating the specific activity of the enzyme.

The specific activity of cholesterol 7α -hydroxylase in liver microsomes from rats maintained on the commercial pellet diet was of the order of 30 pmoles per minute per mg of microsomal protein. If it is assumed that the conversion of cholesterol to 7α -hydroxycholesterol is the only rate-limiting step in the transformation of cholesterol to bile acids, then this rate should give a measure of the rate of formation of bile acids *in vivo*. For a 10 g liver and a microsomal protein content of 12 mg per g of liver microsomes, this *in vitro* enzyme activity corresponds to a rate of formation of approximately 2 mg of bile acids per 24 hours. The rate of formation of total bile acids in rats fed on a commercial diet has been estimated to be approximately 5 mg per 24 hours (Strand 1963). Thus cholesterol 7α -hydroxylase activity measured *in vitro* is of the same order as the rate of formation of bile acids *in vivo*, providing further evidence that this enzyme may be rate-limiting in bile acid synthesis. The discrepancy

between these values could be due to loss in enzyme activity during the preparation, or to a low yield of microsomes per unit liver weight. There is evidence to suggest that other steps in bile acid biosynthesis might become rate-limiting under certain conditions. For example, it has been shown that the enzyme catalysing the 12α-hydroxylation of 7α-hydroxycholest-4-ene-3-one (step D, Fig. 2), which may have a role in regulating the relative proportions of cholic and chenodeoxycholic acids, is activated following starvation (Johansson 1970).

Further evidence that cholesterol 7α-hydroxylase is rate-limiting in bile acid formation comes from the effect of different *in vivo* treatments on the enzyme activity measured *in vitro*. Cholestyramine feeding increased cholesterol 7α-hydroxylase activity, in agreement with previous reports. The enhancement in this case was three to four-fold. Other workers have reported increases in enzyme activity ranging from two-fold (Mitropoulos and Balasubramaniam 1972) to six-fold (Boyd *et al* 1969). The magnitude of the increase probably depends on the control diet on which the animals are maintained. Table 3 demonstrates that cholesterol 7α-hydroxylase activity is influenced by diet. This effect might be caused by a direct action of dietary constituents on bile acid absorption from the gut, as discussed in Section 4 (Part B), but it might also result from an effect of the diet on intestinal micro-organisms. Intestinal bacteria can modify the chemical structure of bile acids. Since certain of these secondary bile acids are only poorly

absorbed from the gut, these will not exert a feedback effect on the enzyme system. Gustafsson *et al* (1957) showed that the half-life of cholic acid was greater in germ-free rats than in conventional rats.

When the effect of various physiological stimuli on the activity of an enzyme measured *in vitro* are investigated, the enzyme must be assayed under conditions of saturating substrate concentration. In the case of the membrane-bound enzyme, cholesterol 7α-hydroxylase, the hydrophobic substrate cholesterol is not presented to the enzyme in true solution and hence the term 'concentration' is not valid. Also, since liver microsomal preparations probably contain many binding sites for cholesterol other than the 'active' site for cholesterol hydroxylation, the kinetics which are normally applied tc enzyme systems in solution cannot be applied to liver microsomal cholesterol 7α-hydroxylase. Microsomal cholesterol probably exists in distinct heterogeneous pools, some of which might be involved in membrane structure and restrained from reacting with the enzyme. If the pool of 'metabolically active' cholesterol does not saturate the enzyme system, then the rate of formation of 7α-hydroxycholesterol might depend on the rate of supply of substrate to this pool. In 1961 Myant and Eder reported that the increase in bile acid biosynthesis in the bile fistula rat was preceded by an increase in the rate of hepatic cholesterol biosynthesis. They suggested that bile acid biosynthesis might be enhanced as a passive consequence of the increased supply of substrate cholesterol. Cholesterol supplied in the diet can also

serve as a precursor of bile acids (Siperstein and Chaikoff 1952). The increase in 7α-hydroxycholesterol formation by liver microsomes from rats fed a high cholesterol diet might also be a consequence of enlargement of a 'substrate pool'. However, the demonstration that the rate of formation of 7α-hydroxycholesterol is first order with respect to microsomal protein concentration suggests that the enzyme is in fact operating under conditions where the substrate is saturating. There is additional evidence to suggest that the rate of supply of substrate cholesterol can be enhanced without affecting the rate of 7α-hydroxycholesterol formation. Tomatine feeding was shown to result in an increased rate of hepatic cholesterol biosynthesis without affecting cholesterol 7α-hydroxylase activity. Tomatine complexes with cholesterol in the gut and prevents it from being absorbed, however it does not interfere with bile acid absorption (Cayen 1971). Cholestyramine feeding, which inhibits the intestinal absorption of both cholesterol and bile acids, was shown to stimulate both hepatic cholesterol biosynthesis and cholesterol 7α-hydroxylase. These results suggest that hepatic cholesterol biosynthesis is under the feedback control of absorbed cholesterol, whereas cholesterol catabolism is controlled by bile acid absorption.

The relative proportions of 'metabolically active' and 'metabolically restrained' cholesterol in liver microsomes are unknown. A possible model for cholesterol compartmentation in liver microsomes is proposed in Fig. 36. Pool 1 represents 'metabolically inactive'cholesterol which is probably

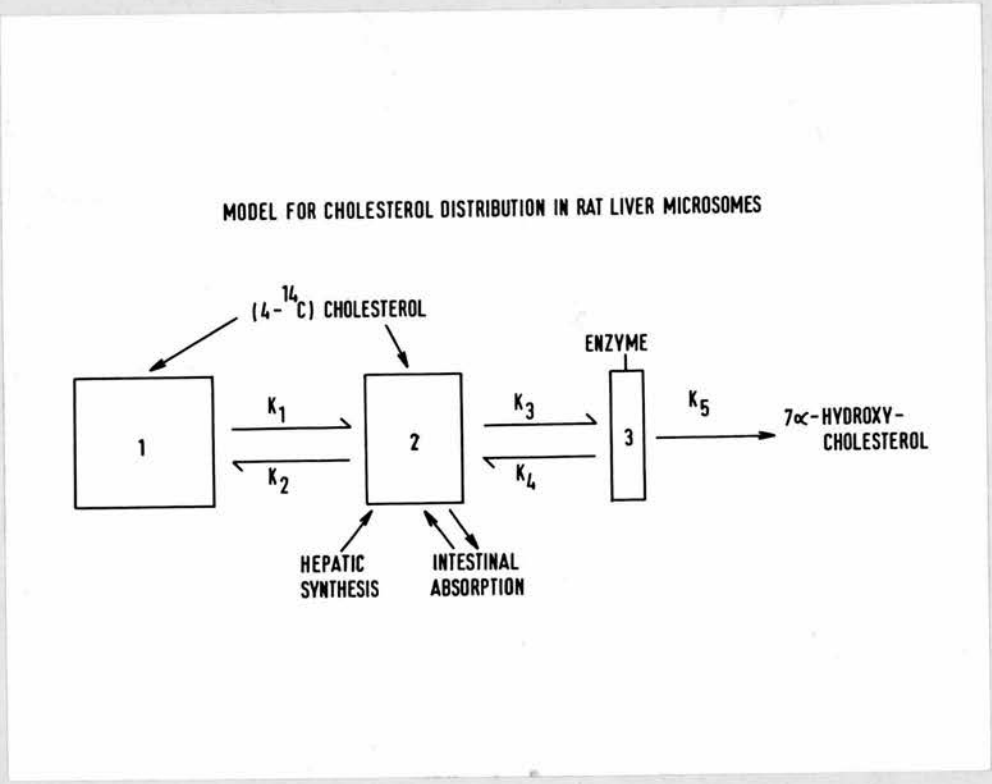

MODEL FOR CHOLESTEROL DISTRIBUTION IN RAT LIVER MICROSOMES
(4-14C) CHOLESTEROL
ENZYME
1
K1
K2
2
K3
K4
3
K5
7α-HYDROXY-CHOLESTEROL
HEPATIC SYNTHESIS
INTESTINAL ABSORPTION

Fig. 36

involved in membrane structure and equilibrates at a slow rate with the 'metabolically active' pool 2. Pool 2 can be converted to bile acids or excreted as faecal neutral sterols, and is replenished by de novo hepatic synthesis and by cholesterol absorbed from the intestine. Exogenous (4-^{14}C) cholesterol added in acetone solution equilibrates equally well with both pools 1 and 2 as demonstrated by the results presented in Section 3 (Part F). Preincubation of (4-^{14}C) cholesterol with liver microsomes prior to starting the reaction with NADPH was found to result in a small but significant increase in (4-^{14}C) 7α-hydroxycholesterol formation. This can be explained by postulating the existence of a third pool of cholesterol, present as the enzyme-substrate complex. Preincubation would then allow (4-^{14}C) cholesterol to equilibrate with this pool, this equilibration proceeding at a slow rate (K_3, Fig. 36). The results shown in Table 13 suggest that this pool represents at most 0.5% of the total endogenous microsomal cholesterol. In these experiments the time for preincubation was 10 minutes, but the half-time for the maximal effect to be obtained was estimated to be approximately 5 minutes. Using this value, the rate of formation of enzyme-cholesterol complex, R, can be calculated for a typical experiment:

$$R = (0.25/100 \times 200)\ \mu g \text{ cholesterol transferred per 5 minutes per 10 mg protein.}$$

$$= 25 \text{ pmoles per minute per mg microsomal protein.}$$

This value is of the same order as the rate of cholesterol 7α-hydroxylation obtained in these experiments. Thus the

rate of formation of enzyme-cholesterol complex might be rate-limiting in cholesterol 7α -hydroxylation.

This could explain the lack of correlation between cholesterol 7α -hydroxylase activity and the cytochrome P450 content of rat liver microsomes. Cytochrome P450 is implicated in cholesterol 7α -hydroxylase as demonstrated by the inhibition of the enzyme by carbon monoxide which is reversed by monochromatic light at 450 nm. The administration of barbiturates, such as phenobarbitone, which induce drug hydroxylase activity also induce liver microsomal cytochrome P450 content and the activity of the flavoprotein NADPH cytochrome c reductase, suggesting that these electron transferring components are rate-limiting (Orrenius et al 1965). However there is no correlation between cytochrome P450 content and cholesterol 7α -hydroxylase activity. Although phenobarbitone increased the total activity of enzyme in the liver there was no change when activity was expressed on the basis of microsomal protein concentration.

Other instances have been recorded where cytochrome P450-dependent hydroxylation was not proportional to cytochrome P450 content. For example, the activity of ethylmorphine demethylase was found to be three-fold greater in male rats than in female rats, whereas there was only a 20% difference in cytochrome P450 content (Gillette and Gram 1969). These workers correlated the difference in enzymic activity with the increase in activity of NADPH cytochrome P450 reductase activity which occurs in the presence of substrate, suggesting that the rate-limiting step in ethylmorphine metabolism is the rate of reduction

of the cytochrome P450-substrate complex. Schenkman et al (1967b) showed that the difference in aminopyrine demethylase activity between male and female rats could be correlated with the magnitude of the Type I spectral change which is produced when the substrate is added to aerobic suspensions of liver microsomes. These workers suggested that the rate-limiting factor was a substrate binding component unique to the cytochrome P450. No sex difference has been observed in cholesterol 7α -hydroxylase activity although a systematic study has not been made. The results presented in Section 5 clearly demonstrate that cholesterol 7α -hydroxylase and aminopyrine demethylase are controlled by different mechanisms in vivo.

Some workers have proposed the existence of a 'cholesterol-specific' cytochrome P450 to explain the lack of correlation between cholesterol 7α -hydroxylase and liver microsomal cytochrome P450 content (Wada et al 1969; Atkin et al 1972). They propose that this cytochrome, rate-limiting in cholesterol 7α -hydroxylation, is present in such a small proportion to the total cytochrome P450 that its induction following biliary diversion is not observed. The difference in sensitivity of different hydroxylases to carbon monoxide was originally interpreted by some workers as being indicative of a plurality of cytochromes which interact with carbon monoxide to different extents. However, Björkhem (1972) has recently correlated the sensitivity of different hydroxylases to carbon monoxide with the presence or absence of an isotope effect in the insertion of thehydroxyl group. For example, the 7α -hydroxylation of taurodeoxycholic acid,

which is insensitive to carbon monoxide, may still involve cytochrome P450, as the rate-limiting step in the reaction is the removal of the hydrogen atom at C_7. No significant isotope effect was observed in the 7α -hydroxylation of cholesterol by rat liver microsomes (Björkhem 1971), suggesting that the breaking of the carbon-hydrogen bond is not rate-limiting in this reaction, perhaps a consequence of the allylic position of C_7 in the cholesterol molecule.

Only two forms of cytochrome P450 have as yet been distinguished in liver microsomes on the basis of the spin state of the haem moiety (Jefcoate et al 1969), but there is no evidence as to whether these can be subdivided into distinct species of cytochrome. Levin and Kuntzmann (1969) showed that the cytochrome P450 of liver microsomes labelled in the haem moiety decayed biphasically in vivo, the half-lives of the two components being 48 hours and 7 hours for the high spin and low spin forms respectively. However the half-life of cholesterol 7α -hydroxylase in vivo was estimated to be approximately 3 hours. Thus although the results can be explained by postulating the existence of a distinct cytochrome P450, they can also be accounted for by proposing the existence of another rate-limiting step, specific for cholesterol. As suggested above, this step could be the formation of an enzyme-cholesterol complex. This might be regulated in turn by a cycloheximide-sensitive factor having a half-life in vivo of 3 hours which is induced following biliary diversion. This rate-limiting component could be a binding protein which holds the cholesterol molecule in the correct conformation at the

active site of the enzyme complex, or it might be a 'carrier protein' for transporting substrate cholesterol through the endoplasmic reticular membranes. Ritter and Dempsey (1971) reported that a heat-stable protein factor isolated from 105,000 g rat liver supernatant stimulated several steps in cholesterol biosynthesis. However this protein, which can bind cholesterol, is probably not involved in cholesterol 7α -hydroxylase, as heat-stable supernatant from the livers of cholestyramine-treated rats had no effect on cholesterol 7α -hydroxylase activity in liver microsomes from control rats (Scholan 1969).

Provided that the rate-limiting component of cholesterol 7α -hydroxylase is saturated, then the enzyme will operate under Vmax conditions, regardless of whether the hydroxylation site on the P450 is saturated with cholesterol. It is interesting to note that the specific activity of aminopyrine demethylase is two orders of magnitude greater than the specific activity of cholesterol 7α -hydroxylase. It is possible that the hydroxylation of cholesterol by cytochrome P450 proceeds at an equally fast rate, but that the overall rate is determined by the rate at which cholesterol is transferred from pool 2 to pool 3 (Fig. 36). This hypothesis necessitates an explanation as to why cholesterol does not interact directly with the cytochrome P450 but requires in addition another component. This could be a consequence of the hydrophobicity of the cholesterol molecule. Table 14 demonstrates that pregn-5-ene-3β -ol did not produce a difference spectrum with rat liver microsomes whereas dehydroepiandrosterone, pregnenolone and

androst-5-ene-3β-ol all produced Type I difference spectra. Since pregn-5-ene-3β-ol differs from pregnenolone in the lack of a keto group at C_{20}, and from androst-5-ene-3β-ol in the 2-carbon side-chain at C_{17}, it appears that there is a limit to the hydrophobicity of substrates which can interact with cytochrome P450. Thus cholesterol, having an 8-carbon side chain at C_{17} may be structurally unsuited to interact directly with cytochrome P450 and therefore requires an additional component. The specificity of this component for cholesterol is demonstrated by the results obtained on the NADPH-dependent oxidation of steroid substrates having a structure similar to cholesterol. The oxidation of these substrates was not affected by cholestyramine feeding, a treatment which induces cholesterol 7α-hydroxylase activity. It is possible that dehydroepiandrosterone, pregnenolone and androst-5-ene-3β-ol can interact directly with cytochrome P450. The rates of oxidation of these substrates, like that of aminopyrine demethylase, were two orders of magnitude greater than cholesterol 7α-hydroxylase activity. These compounds probably inhibit the hydroxylation of cholesterol by competing for reducing equivalents from the mixed function oxidase system, however the activating effect of aminopyrine remains unexplained. The results suggest that pregn-5-ene-3β-ol is not oxidised by a mechanism involving either cytochrome P450 or cholesterol 7α-hydroxylase, but that a peroxidative mechanism is involved, analogous to the 'autoxidation' of cholesterol by liver microsomes. Both of these systems are markedly inhibited by β-mercaptoethylamine.

The hypothesis of a rate-limiting factor for cholesterol 7α -hydroxylase, distinct from the electron transferring components of the mixed function oxidase system, could be tested by the fractionation of these components from liver microsomes followed by reconstitution of enzyme activity, however attempts at solubilisation and fractionation of the cholesterol 7α -hydroxylase enzyme system have so far met with little success.

The proposed control mechanism for cholesterol 7α -hydroxylase is similar to that postulated for the mechanism of action of adrenocorticotrophic hormone (ACTH) on the cholesterol side chain cleavage enzyme of adrenal cortex mitochondria (Simpson et al 1972). This system also involves interaction of substrate cholesterol with a membrane-bound mixed function oxidase system of which cytochrome P450 is the terminal oxidase (Simpson and Boyd 1967). Ether anaesthesia stress increases the rate of formation of pregnenolone from cholesterol by adrenal cortex mitochondria in vitro, and evidence has accumulated which suggests that the rate-determining step in this system is the rate of formation of an enzyme-cholesterol complex. In this system cholesterol forms a high spin complex with cytochrome P450, and hence the amount of cholesterol present as enzyme-substrate complex can be quantitated, either by using electron spin resonance techniques, or by measuring the extent of pregnenolone binding (Simpson et al 1972; Jefcoate et al 1972). Pregnenolone forms a Type II complex with adrenal cortex mitochondrial cytochrome P450, interpreted as being a result of

displacement of enzyme-bound cholesterol, with resultant conversion of the cytochrome from the high spin to the low spin state. The product of liver microsomal cholesterol 7α -hydroxylase, 7α -hydroxycholesterol, although it inhibits the enzyme,does not produce a difference spectrum with liver microsomes. This suggests that either product inhibition does not occur as a result of a displacement of substrate from cytochrome P450, or that cholesterol does not form a high spin complex with liver microsomal cytochrome P450 as it does with adrenal mitochondrial cytochrome P450. This latter explanation is supported by the finding that induction of cholesterol 7α -hydroxylase activity is not paralleled by a change in the proportion of high spin cytochrome P450 as measured by amine binding techniques (Grimwade 1971).

Cholesterol metabolism in adrenal cortex also resembles that in liver microsomes in being regulated by a cycloheximide-sensitive factor (Garren et al 1965). However, in the former case the half-life of this factor is of the order of several minutes, as opposed to 3 hours for cholesterol 7α -hydroxylase, in keeping with the different physiological functions of these two systems. A further similarity between cholesterol metabolism in these two organs is the increased rate of substrate supply which accompanies enhancement of substrate degradation in response to physiological stimuli. Thus biliary diversion results in an increase in the rate of hepatic cholesterol synthesis and stress results in an increase in the rate of cholesterol ester hydrolysis in rat adrenal cortex cells

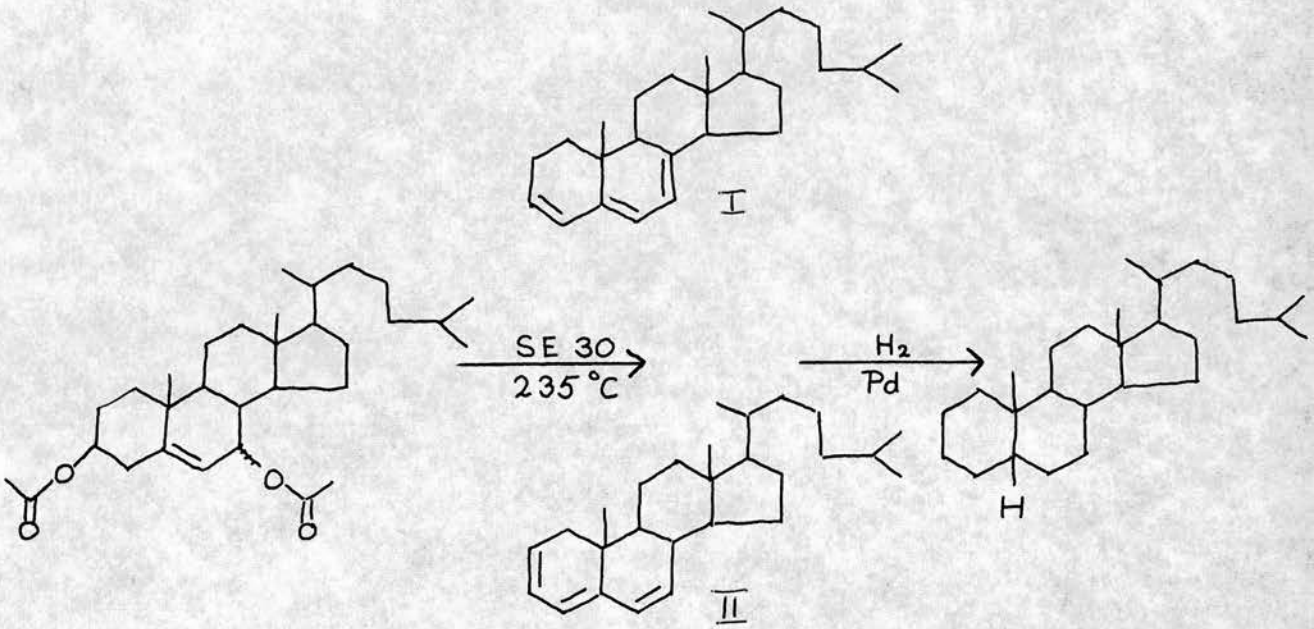
(W. H. Trzeciak, personal communication). Thus in these two organs, each having very different metabolic functions, the control mechanisms which have evolved for cholesterol metabolism are remarkably similar. The enzymic control of cholesterol metabolism in ovary and testis still remain to be elucidated.

APPENDIX I

THE DECOMPOSITION PRODUCT OF CHOLEST-5-ENE-3β,7α-DIOL DIACETATE ON GAS LIQUID CHROMATOGRAPHY

Cholest-5-ene-3β, 7α-diol diacetate, when subjected to gas liquid chromatography under the conditions described in Section 2, resulted in a chromatogram having a single symmetrical peak with retention time relative to cholesterol of 0.64. However, Van Lier and Smith (1967) reported a retention time relative to cholesterol of 1.69 for this compound using the same liquid phase SE 30. The discrepancy between these values suggested that perhaps decomposition was taking place in our experiments. This was confirmed by the following procedure.

Combined gas chromatography-mass spectrometry using the same gas chromatographic conditions as above showed the eluted compound to have a molecular ion of m/e 366. Cholest-5-ene-3β, 7α-diol diacetate has a molecular weight of 486. However, this in itself was not indicative of gas chromatographic decomposition as direct injection of cholest-5-ene-3β, 7α-diol diacetate into the mass spectrometer also yielded a spectrum with a molecular ion of m/e 366. However, when the eluted component was passed over 'neutral' palladium using hydrogen as the gas chromatographic carrier gas prior to entry into the mass spectrometer, the spectrum obtained showed a molecular ion of m/e 372. This spectrum was identical with that of 5β-cholestane. This evidence indicates that cholest-5-ene-3β, 7α-diol diacetate has been decomposed following injection into the gas chromatograph to give a cholestatriene.



cholest-5-ene-3β, 7α-diol diacetate

5β-cholestane

Suggested structures for the decomposition product are $\Delta^{3,5,7}$-cholestatriene (I) which could be formed by the thermal elimination of two molecules of acetic acid, or the rearrangement product $\Delta^{2,4,6}$-cholestatriene (II). The identity of this cholestatriene was not established.

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